AN OPEN LABEL TWO WAY TWO PERIOD RANDOMISED SINGLE DOSE COMPARITIVE ORAL BIOAVAILABILITY STUDY OF ZAFIRLUKAST IN HEALTHY VOLUNTEERS UNDER FASTING CONDITIONS

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IN

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By

Register No: 261425015

Under the Guidance of

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All India Council for Technical Education, New Delhi.

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CERTIFICATE

This is to certify that the project entitled, **AN OPEN LABEL TWO WAY TWO PERIOD RANDOMISED SINGLE DOSE COMPARITIVE ORAL BIOAVAILABILITY STUDY OF ZAFIRLUKAST IN HEALTHY VOLUNTEERS UNDER FASTING CONDITIONS** by **Reg. No. 261425015 ,** submitted in partial fulfillment for the degree **Master of Pharmacy (Pharmacology)** was carried out at the Dept. of Pharmacology, C. L. Baid Metha College of Pharmacy, Chennai-97 under my supervision during the academic year 2015-2016.

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Date: **(Prof. Dr. GRACE RATNAM)**

Place: Chennai

DECLARATION

I, Register No. **261425015 ,** hereby declare that this dissertation entitled, INVIVO BIOEQUIVALENCE STUDY OF ANTIASTHMATIC DRUG ZAFIRLUKAST IN HEALTHY VOLUNTEERS**,** has been originally carried out by me under the guidance and supervision of Prof. **Dr. P. Muralidharan**, M. Pharm, PhD, Head, Dept. of Pharmacology, C. L. Baid Metha College of Pharmacy, Chennai-97, for the academic year 2015-2016. This work has not been submitted in any other degree at any other university.

Register No.

Date:

Place:

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At last, never the least I am greatly thankful to my friends, family and all well-wishers for their moral support.

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LIST OF ABBREVIATIONS

ANOVA	:	analysis of variance
API	:	atmospheric pressure ionization
AUC	:	area under curve
AUC Extrapolated (%)	:	percent area under plasma conc extrapolated from
$AUC_{0-\alpha}$	:	.the area under curve from time zero to infinity
AUC_{0-t}	:	the area under curve from timezero to last measurable conc
BA	:	bioavailability
BE	:	bioequivalence
BMI	:	body mass index
CC	:	calibration curve
CEC	:	central ethics committee
CI	:	confidence interval
C_{max}	:	maximum observed drug conc
CV	:	coefficient of variance
D	:	day
DEPT	:	department
DF	:	degrees of freedom
ECG	:	electrocardiogram

F-value	:	fishers ‘f’ distribution value
GCP	:	good clinical practice
GLP	:	good laboratory practice
HPLC	:	high performance liquid chromatography
HQC	:	high quality control
ICF	:	informed consent form
ICH	:	international conference on harmonization
IP	:	investigational product
K_{el}	:	elimination rate constant
K_3 EDTA	:	tri potassium ethylene diamine tetra acetic acid
Lambda Z	:	K_{el}
LC-MS/MS	:	liquid chromatography – mass Spectrometry
Ln	:	logarithm to the base ‘e’ or (natural logarithm)
LQC	:	low quality control
LSM	:	least square mean
m/z	:	mass to charge ratio
mL	:	milli litre
mM	:	millimolar

MQC	:	medium quality control
N	:	no of subjects
PI	:	principal investigator
Pk parameter	:	pharmacokinetic parameter
PD	:	pharmacodynamics
Prob	:	probability value (p-value)
Residual area (%)	:	AUC extrapolated (%)
RPM	:	rotations per minute
R^2	:	regression coefficients
SAS	:	statistical analysis software
SD	:	standard deviation
STD	:	standard
Subject (seq)	:	subject nested within sequence
$T_{1/2}$	:	estimated terminal half life ($T_{1/2}$)
T_{max}	:	time corresponding to C_{max}
V/V	:	volume by volume
^0C	:	degree centigrade
µg	:	microgram
µg/ml	:	microgram/ milliliter
µL	:	microlitre

INTRODUCTION

Generic drugs are cost effective alternatives for the brand name drugs and the savings are estimated in the average $8 to $10 billion a year. (Lauren et al., 2009; Information for Consumers). Over the years the prescription of generic drugs has increased substantially (1984: 19% & 2009- 60-70%) (**IMS health; Information for consumers**). Bioequivalence testing is playing a vital role in generic drug development. The generics have to be developed and tested in human subjects by following stringent GCP/GLP standards. From industry point of view, there is a need to conduct bioequivalence studies at an allowable cost to have an effective generic development program in a scientifically acceptable standard. In order to achieve this from time to time various regulatory agencies have issued guidance's to bring more clarity and uniformity for conducting Bioavailability (BA)/ Bioequivalence (BE) studies.

Pharmaceutical companies develop products based on their business plans and development of generics for USA and EU gets the priority. In general FDA suggests highest strength as RLD or as per individual product recommendations where as for EU generally highest strength or the choice should be justified if lower strength is used based on safety, linearity and dose proportionality, but for selection of dose and strength one need to depend on the literature but lack and validity of the literature (NfG on the Investigation of Bioequivalence- CPMP/QWP/EWP/1401/98 Rev. 1).

As per the present scenario generic product development by the entrepreneur is carried out for all the markets simultaneously in order to reduce cost. Recently EU has come out with a relatively better option to reduce the developmental cost of generic product like same test product can be compared against two references in a 3 way design, but in case of failure with any one of the innovator, industry has to go back to reformulation (NfG on the Investigation of Bioequivalence- CPMP/QWP/EWP/1401/98 Rev. 1).

Development of ANDAs for highly variable drug is the major concern for the generic drug industry. Drugs and drug products that exhibit high within-subject variability in Cmax and AUC present a challenge for the design of BE studies. For example, a drug with a variability of 50%

would require a study in 100 subjects to demonstrate the equivalence of the reference to itself. So development of study designs that would allow demonstration of bioequivalence with a smaller number of subjects was needed. (Marier et al., 2008)

Draft EMEA guidance (CHMP- Guideline on the investigation of bioequivalence; CPMP/EWP/QWPI1401198 Rev.1:2012) says,
"In case the pro-drug or active metabolites display non-linear pharmacokinetics, it is recommended to demonstrate bioequivalence for the main active metabolite. In such case, the parent compound does not need to be measured provided that it is inactive from efficacy and safety perspectives." Based on this studies were conducted on valacyclovir by measuring aciclovir. But as per the final guidance EMEA (NfG on the Investigation of Bioequivalence-CPMP/\ QWP/EWP/1401/98 Rev. 1), we need to measure parent compound Valaciclovir[1].

The BE studies should normally be performed in healthy volunteers unless safety warranties. Study in healthy volunteers, is adequate to detect formulation differences and allow extrapolation of the results to populations for which the reference product is approved (theelderly, children, patients with renal or liver impairment, etc.). There is a wide experience that two formulations that were
bioequivalent in one study population will also be bioequivalent in other populations (Rhodes, 1995). Generic drug developers are still behind the exact reason for proving BE in special population, particularly when it is a crossover design.
A generic equivalent drug product may be marketed by a drug company only after proving that it is bioequivalent to the innovator product. This is true whether a generic company wants to register its drug with USFDA and sell it in USA or if it wants to sell it in India.

As per Food-effect Bioavailability and Fed Bioequivalence guideline

"In addition to a BE study under fasting conditions, we recommend a BE study under fed conditions for all orally administered immediate release drug products, with the following exceptions:

• When both test product and RLD are rapidly dissolving, have similar dissolution profiles, and Biopharmaceutics Classification System (BCS) Class I or

• When the DOSAGE AND ADMINISTRATION section of the RLD label states that the product should be taken only on an empty stomach, or

• When the RLD label does not make any statements about the effect of food on absorption or administration." (Food-Effect: Guidance for Industry, 2002)

If we tweak our objective that in the global marketplace, all generic, multisource, drug products should be bioequivalent and therapeutic equivalent to a single, standard RLD to avoid possible significant variations among generic drugs and their brand name counterpart, it could possibly reduce the burden of generic entrepreneur. But in order to achieve it we need to come out with a universal reference (Leon et al., 2009).

1.0 General introduction

1.1 BIOAVAILABILITY

To exert an optimal therapeutic action an active moiety should be delivered to its site of action in an effective concentration for the desired period. To allow reliable prediction of the therapeutic effect the performance of the dosage form containing the active substance should be well characterized.

Bioavailability (BA) studies focuses on the process by which the active ingredients or moieties are released from an oral dosage form and move to the site of action. BA data provide an estimate of the fraction of the drug absorbed, as well as its subsequent distribution and elimination.

BA can be generally documented by a systemic exposure profile obtained by measuring drug and/or metabolite concentration in the systemic circulation over time. The systemic exposure profile determined during clinical trials in the Investigational New Drug (IND) period can serve as a benchmark for subsequent BE studies.

Bioavailability is defined as the rate and extent to which the active ingredient, or active moiety, is absorbed from a drug product, and becomes available at the site of action. For drug products that are not intended to be absorbed into the bloodstream, bioavailability may be assessed by measurements intended to reflect the rate and extent to which the active ingredient or active moiety becomes available at the site of action.

From a pharmacokinetic (PK) perspective, BA data for a given formulation provides an estimate of the relative fraction of the orally administered dose that is absorbed into the systemic circulation when compared to the BA data for a solution, suspension, or intravenous dosage form. In addition, BA studies provide other useful pharmacokinetic information related to distribution, elimination, the effects of nutrients on absorption of the drug, dose proportionality, linearity in pharmacokinetics of the active moieties and, where appropriate, inactive moieties. BA data may also provide information indirectly about the properties of a drug substance before entry into the systemic circulation, such as permeability and the influence of presystemic enzymes and/or transporters (e.g., p-glycoprotein).

Systemic exposure patterns reflect both release of the drug substance from the drug product and a series of possible presystemic/systemic actions on the drug substance after its release from the drug product.

Comparative studies are performed to understand the relative contribution of these processes to the systemic exposure pattern.

Comparison of therapeutic performances of two medicinal products containing the same active substance is a critical means of assessing the possibility of alternative use between the innovator and any essentially similar medicinal product. Assuming that in the same subject an essentially similar plasma concentration time course will result in essentially similar concentrations at the site of action and thus in an essentially similar effect, pharmacokinetic data instead of therapeutic results may be used to establish equivalence – bioequivalence[2].

In vivo performance, in terms of BA/BE, may be considered to be one aspect of product quality that provides a link to the performance of the drug product used in clinical trials, and to the database containing evidence of safety and efficacy.

Studies to measure BA and/or establish BE of a product are important elements in support of Investigational New Drugs (INDs), New Drug Applications (NDAs), Abbreivated New Drug Applications (ANDAs), and their supplements.

1.2 BIOEQUVILANCE

Bioequivalence gained increasing attention during the last 40 years after it became evident that generic product and innovator product, having the same amounts of the drug, may exhibit marked differences in their therapeutic responses.

Nowadays Bioequivalence studies are a pivotal part of registration dossiers. These studies measure the bioavailability of two (or more) formulations of the same active ingredient. The purpose of the study is that the bioavailability of the formulations under investigation is shown to be equal. Based on that conclusion, one may subsequently claim that the therapeutic quality of these formulations is identical. The latter means that both the beneficial and side effects are identical and hence the formulations are interchangeable.

Bioequivalence studies compare both the rate and extent of absorption of various multisource formulations with the innovator (reference) product, on the basis that if two formulations exhibit similar drug concentration-time profiles in the blood/plasma, they should exhibit similar therapeutic effects.

Bioequivalent simply means that one brand or dosage form of a drug or supplement is equivalent to a reference brand or dosage form of the same drug or supplement in terms of various bioavailability parameters measured via in vivo testing in human subjects. A product can be either bio-equivalent or bio-in equivalent. A product cannot be more bio-equivalent or less bio-equivalent.

Two medicinal products containing the same active substance are considered bioequivalent if they are pharmaceutically equivalent or pharmaceutical alternatives and their bioavailabilities (rate and extent) after administration in the same molar dose lie within acceptable predefined limits. These limits are set to ensure comparable *in vivo* performance, i.e. similarity in terms of safety and efficacy.

In bioequivalence studies, the plasma concentration time curve is generally used to assess the rate and extent of absorption. Selected pharmacokinetic parameters and preset acceptance limits allow the final decision on bioequivalence of the tested products.

- AUC, the area under the concentration time curve, reflects the extent of exposure.
- Cmax, the maximum plasma concentration or peak exposure, and
- Tmax, the time to maximum plasma concentration, are parameters that are influenced by absorption rate. (EMEA)

Bioequivalence is defined as:

the absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriately designed study.

On the basis of simple pharmacokinetic concepts and parameters, bioavailability and bioequivalence studies have been established as acceptable surrogates for expensive, complicated and lengthy clinical trials, and are used extensively worldwide to establish and ensure consistent quality and a reliable, therapeutically effective performance of marketed dosage forms.

1.3 Role of BE in drug development

1. IND (INVESTIGATIONAL NEW DRUG)/NDAs(NEW DRUG APPLICATIONS

BE documentation can be useful during the IND or NDA period to establish links between (1) early and late clinical trial formulations; (2) formulations used in clinical trial and stability studies, if different; (3) clinical trial formulations and to-be-marketed drug product; and (4) other comparisons, as appropriate.

In each comparison, the new formulation or new method of manufacture is the test product and the prior formulation or method of manufacture is the reference product.

A test product can fail to meet BE limits because the test product has higher or lower measures of rate and extent of absorption compared to the reference product or because the performance of the test or reference product is more variable.

2. ANDAs (ABBRIEVATED NEW DRUG APPLICATIONS)

BE studies are a critical component of ANDA submissions. The purpose of these studies is to demonstrate BE between a pharmaceutically equivalent generic drug product and the corresponding reference listed drug. Together with the determination of pharmaceutical equivalence, establishing BE allows a regulatory conclusion of therapeutic equivalence.

3. Postapproval Changes

For approved NDAs, we also recommend that the drug product after the change be compared to the drug product before the change. For approved ANDAs, we also recommend that the drug product after the change be compared to the reference listed drug.

Three situations have been defined in which bioequivalence studies are required

- When the proposed marketed dosage form is different from that used In pivotal clinical trials,
- When significant changes are made in the manufacture of the marketed formulation, and
- When a new generic formulation is tested against the innovator's marketed product.

Bioequivalence studies impact of changes to the dosage form process after pivotal studies commence to ensure product on the market is comparable to that upon which the efficacy is based

- Establish that a new formulation has therapeutic equivalence in the rate and extent of absorption to the reference drug product.
- Important for linking the commercial drug product to clinical trial material at time of NDA
- Important for post-approval changes in the marketed drug formulation

Once bioequivalence is established between two products the concept of prescribability and switchability comes into picture. These are concepts about the ease with which the physician can prescribe the generic product or the innovator product depending on various factors.

Drug prescribability is defined as the physician's choice for prescribing an appropriate drug product for his/her new patients between a brand-name drug product and a number of generic drug products of the brand-name product, which have been shown to be bioequivalent to the brand-name drug product. The underlying assumption of drug prescribability is that the brand-name drug product and its generic copies can be used interchangeably in terms of the efficacy and safety of the drug product.

Drug switchability is related to the switch from a drug product (eg, a brand-name drug product) to an alternative drug product (eg, a generic copy of the brand-name drug product) within the same subject whose concentration of the drug product has been titrated to a steady, efficacious, and safe level. As a result, drug switchability is considered more critical than drug prescribability in the study of drug interchangeability for patients who have been on medication for a while. To assure drug switchability, it is recommended that bioequivalence be assessed within individual subjects. This type of bioequivalence is known as individual bioequivalence (IBE).

New drug

A new drug means and include

(a) A drug, as defined in the Act including bulk drug substance which has not been used in the country to any significant extent under the conditions prescribed, recommended or suggested in the labelling thereof and has not been recognized as effective and safe by the licensing authority.

(b) A drug already approved by the Licensing Authority for certain claims, which is now proposed to be marketed with modified or new claims, namely, indications, dosage, dosage form (including sustained release dosage form) and route of administration.

(c) A fixed dose combination of two or more drugs, individually approved earlier for certain claims, which are now proposed to be combined for the first time in a fixed ratio, or if the ratio of ingredients in an already marketed combination is proposed to be changed, with certain claims, viz. indications, dosage, dosage form (including sustained release dosage form) and route of administration.

(i) All vaccines shall be new drugs unless certified otherwise by the Licensing Authority.
(ii) A new drug shall continue to be considered as new drug for a period of four years from the date of its first approval or its inclusion in the Indian Pharmacopoeia, whichever is earlier.

Generic drug

Generic drugs are safe and effective alternatives to brand name prescriptions. Generic drugs can help both consumers and the government to reduce the cost of prescription drugs.

The term "generic product" it means a pharmaceutical product, usually intended to be interchangeable with the innovator product, which is usually manufactured without a license from the innovator company and marketed after expiry of patent or other exclusivity rights.

Pharmaceutical equivalents means drug products that contain identical amounts of the identical active drug ingredient, i.e., the same salt or ester of the same therapeutic moiety, in identical dosage forms, but not necessarily containing the same inactive ingredients, and that meet the identical ingredients or other applicable standard of identity, strength, quality, and purity, including potency and, where applicable, content uniformity, disintegration times and/or dissolution rates.

Pharmaceutical alternatives means drug products that contain the identical therapeutic moiety, or its precursor, but not necessarily in the same amount or dosage form or as the same salt or ester. Each such drug product individually meets either the identical or its own respective compendia or other applicable standard of identity, strength, quality, and purity, including potency and, where applicable, content uniformity, disintegration times and/or dissolution rates.

Innovator product

The innovator product is that which was authorized for marketing (normally as a patented drug) on the basis of documentation of efficacy, safety and quality (according to contemporary requirements).

- No clinical studies have been performed in patients with the Generic Product to support its Efficacy and Safety.
- With data to support similar in vivo performance (= Bioequivalence) efficacy and safety data can be extrapolated from the innovator product to the generic product[3].

1.4 CLINICAL TRIAL

"Clinical trial" means a systematic study of new drug(s) in human subject(s) to generate data for discovering and/or verifying the clinical, pharmacological (including pharmacodynamic and pharmacokinetic) and /or adverse effects with the objective of determining safety and / or efficacy of the new drug.

PHASES OF CLINICAL TRIAL

Human Pharmacology (Phase I):

(i) The objective of studies in this Phase is the estimation of safety and tolerability with the initial administration of an investigational new drug into human(s). Studies in this Phase of development usually have non-therapeutic objectives and may be conducted in healthy volunteers subjects or certain types of patients. Drugs with significant potential toxicity e.g. cytotoxic drugs are usually studied in patients**.** Phase I trials should preferably be carried out by Investigators trained in clinical pharmacology with access to the necessary facilities to closely observe and monitor the Subjects.

(ii) Studies conducted in Phase I, usually intended to involve one or a combination of the following objectives:-

(a) ***Maximum tolerated dose***: To determine the tolerability of the dose range expected to be needed for later clinical studies and to determine the nature of adverse reactions that can be expected. These studies include both single and multiple dose administration.

(b) ***Pharmacokinetics***, i.e., characterization of a drug's absorption, distribution, metabolism and excretion. Although these studies continue throughout the development plan, they should be performed to support formulation development and determine pharmacokinetic parameters in different age groups to support dosing recommendations.

(c) ***Pharmacodynamics****:* Depending on the drug and the endpoints studied, pharmacodynamic studies and studies relating to drug blood levels (pharmacokinetic/ pharmacodynamic studies) may be conducted in healthy volunteer Subjects or in patients with the target disease. If there are appropriate validated indicators of activity and potential efficacy, pharmacodynamic data obtained from patients may guide the dosage and dose regimen to be applied in later studies.

(d) ***Early Measurement of Drug Activity***: Preliminary studies of activity or potential therapeutic benefit may be conducted in Phase I as a secondary objective. Such studies are generally performed in later Phases but may be appropriate when drug activity is readily measurable with a short duration of drug exposure in patients at this early stage.

Therapeutic exploratory trials (Phase II):

(i) The primary objective of Phase II trials is to evaluate the effectiveness of a drug for a particular indication or indications in patients with the condition under study and to determine the common short-term side-effects and risks associated with the drug. Studies in Phase II should be conducted in a group of patients who are selected by relatively narrow criteria leading to a relatively homogeneous population. These studies should be closely monitored. An important goal for this Phase is to determine the dose(s) and regimen for Phase III trials. Doses used in Phase II are usually (but not always) less than the highest doses used in Phase I.

(ii) Additional objectives of Phase II studies can include evaluation of potential study endpoints, therapeutic regimens (including concomitant medications) and target populations (e.g. mild versus severe disease) for further studies in Phase II or III. These objectives may be served by exploratory analyses, examining subsets of data and by including multiple endpoints in trials.

(iii) If the application is for conduct of clinical trials as a part of multi-national clinical development of the drug, the number of sites and the patients as well as the justification for undertaking such trials in India shall be provided to the Licensing Authority.

Therapeutic confirmatory trials (Phase III):

(i) Phase III studies have primary objective of demonstration or confirmation of therapeutic benefit(s). Studies in Phase III are designed to confirm the preliminary evidence accumulated in Phase II that a drug is safe and effective for use in the intended indication and recipient population. These studies should be intended to provide an adequate basis for marketing approval. Studies in Phase III may also further explore the dose-response relationships (relationships among dose, drug concentration in blood and clinical response), use of the drug in wider populations, in different stages of disease, or the safety and efficacy of the drug in combination with other drug(s).

(ii) For drugs intended to be administered for long periods, trials involving extended exposure to the drug are ordinarily conducted in Phase III, although they may be initiated in Phase II. These studies carried out in Phase III complete the information needed to support adequate instructions for use of the drug (prescribing information).

(iii) For new drugs approved outside India, Phase III studies need to be carried out primarily to generate evidence of efficacy and safety of the drug in Indian patients when used as recommended in the prescribing information. Prior to conduct of Phase III studies in Indian subjects, Licensing Authority may require pharmacokinetic studies to be undertaken to verify that the data generated in Indian population is in conformity with the data already generated abroad.

(iv) If the application is for the conduct of clinical trials as a part of multi-national clinical development of the drug, the number of sites and patients as well as the justification for undertaking such trials in India should be provided to the Licensing Authority along with the application.

Post Marketing Trials (Phase IV):

Post Marketing trials are studies (other than routine surveillance) performed after drug approval and related to the approved indication(s). These trials go beyond the prior demonstration of the drug's safety, efficacy and dose definition. These trials may not be considered necessary at the time of new drug approval but may be required by the Licensing Authority for optimizing the drug's use. They may be of any type but should have valid scientific objectives. Phase IV trials include additional drug-drug interaction(s), dose-response or safety studies and trials designed to support use under the approved indication(s), e.g. mortality/morbidity studies, epidemiological studies etc

2.0 AN OVERVIEW ABOUT THE DRUG AND ITS USE

2.1 ASTHMA

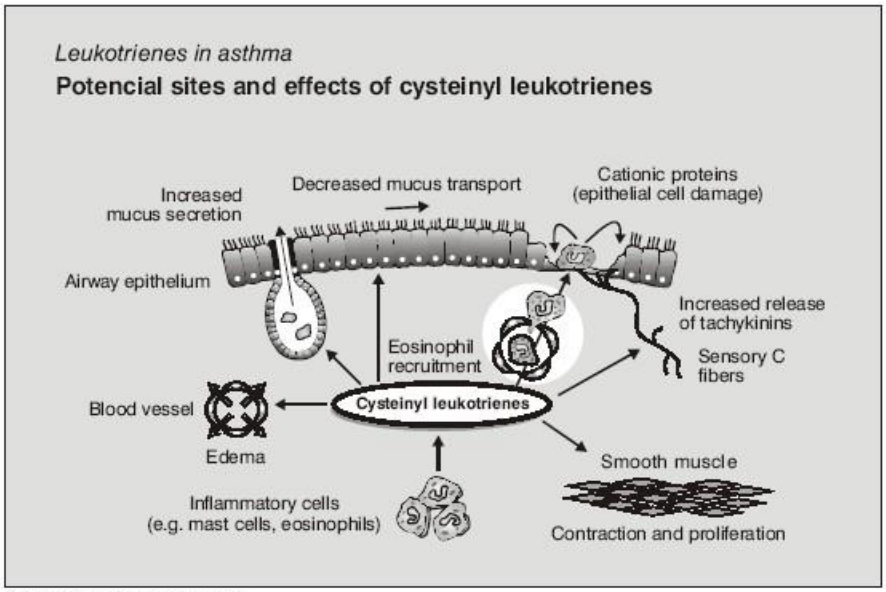
Asthma is defined as "a chronic inflammatory disorder of the airways by the Global Initiative for Asthma[8]. Bronchial asthma is characterized by hyperresponsiveness of tracheobronchial smooth muscle to a variety of stimuli, resulting in narrowing of air tubes, accompanied by increasing secretion, mucosal edema and mucus plugging. Symptoms include dyspnoea, wheezing, and cough.

Asthma was recognized to be a primarily inflammatory condition: inflammation underlying hyper reactivity. A variety of trigger factors are involved. Common asthma triggers includes Dust, cold weather, Chemicals in the air or in food, Exercise, Mold, Pollengrains, Respiratory infections such as the common cold, Strong emotions (stress),Tobacco smoke, pet hair.

Asthma is considered as

- Extrinsic asthma: it is mostly episodic, less prone to status asthmaticus.
- Intrinsic asthma: it tends to be perennial, status asthmaticus is more common.

Mediators like mast cells, eosinophils and inflammatory cells together constrict bronchial smooth muscle, cause mucosal edema and all resulting in reversible airway obstruction. Majority of asthmatics are atopic. In such atopic subjects, challenge of the airways with allergens to which they are sensitive leads to bronchoconstriction[5].



Adapted from Hay DW, et al.

Leukotriene actions on airway structures

Clinical classification of severity					
Severity in patients ≥ 12 years of age	**Symptom frequency**	**Night time symptoms**	**%FEV_1 of predicted**	**FEV_1Variability**	**Use of short-acting $beta_2$ agonist for symptom control (not for prevention of EIB)**
Intermittent	≤2 per week	≤2 per month	≥80%	<20%	≤2 days per week
Mild persistent	>2 per week but not daily	3-4 per month	≥80%	20–30%	>2 days/week but not daily
Moderate persistent	Daily	>1 per week but not nightly	60–80%	>30%	Daily
Severe persistent	Throughout the day	Frequent (often 7x/week)	<60%	>30%	Several times per day

Bronchial asthma is clinically divided as[6],

1. Mild intermittent asthma:
   This is often a recognizable precipitating factor such as allergy, an upper respiratory tract infection or psychological trauma.
2. Chronic persistent asthma:
   This is generally due to presence of inflammation and thickening of mucosa of bronchioles with excessive secretion of mucus. The chronic is divide into mild, moderate and severe depending on the interference with dialy activities. In some chronic asthma co-exists with COPD.
3. Severe acute asthma:
   It is a condition where acute asthma is severe, persistent, it is accompanied by evidence of respiratory insufficiency or failure.
4. Exercise-induced asthma:
   In this attack is precipitated by exercise or inhalation of cold air.

3.2 TYPES OF ASTHMA

Brittle asthma

It is two types of asthma, noticed by recurrent, severe attacks.

- Type 1 brittle asthma shows wide peak flow variability, intense medication.
- Type 2 brittle asthma associated by well-controlled asthma, with sudden severe exacerbations.

Asthma attack

It is an acute asthma exacerbation. shortness of breath, wheezing, and chest tightness are the classical symptoms.

The use of accessory muscles (sternodeidomastoid and scalene muscles) of respiration produces a pulse that is weaker during inhalation and stronger during exhalation and over-inflation of the chest are the symptoms of acute asthma attack.

The peak expiratory flow rate (PEFR) shows in case of

- In a mild exacerbation it is $\geq$ 200 L/min or $\geq$ 50%
- In a moderate it is between 80 and 200 L/min or 25% and 50%.
- In a severe it is $\leq$ 80L/min or $\leq$ 25%.

Status asthmaticus

It is an acute exacerbation of asthma which does not respond to standard treatments of bronchodilators and steroids. Nonselective beta blockers (eg: Timolol) caused fatal status asthmaticus.

Exercise induced

The asthma which is common among top athletes.

It is relatively high incidence of asthma occurs in sports such as

- mountain biking,
- cycling,
- long-distance.

Occupational

Asthma which shows or associated with the workplace exposures is a commonly known occupational asthma or occupational respiratory disorder.

Generally 15–23% of asthma cases in adults are work related according to American thoracic society (2004). The occupation like fabricators, operators shows high percentage of work related asthma. The manufacturing industries are generally associated with these cases. Others like animal proteins, natural rubber, and certain reactive chemicals also causes occupational asthma[7].

PRINCIPLES OF THERAPY:

Generally treatment include

- Relieving bronchospasm
- Reducing the inflammation

The available therapeutic measures are:

- Elimination of trigger factors: eg., allergens, environmental pollution
- Avoiding respiratory irritants: eg., smoking
- Drug therapy: use of bronchodialators and anti-inflammatory drugs
- Correction of dehydration and acidosis in severe acute attack.
- Controlled administration of oxygen
- Physical exercise
- Psychological treatment.

3.3 EPIDEMIOLOGY

Asthma is major health problem across worldwide. In developed and westernized countries show high rates of asthma. Asthma shows 7-10% across world wide. Symptoms were more prelevant in United Kingdom, Australia, Newzealand and lowest in eastern Europe, Greece, India.

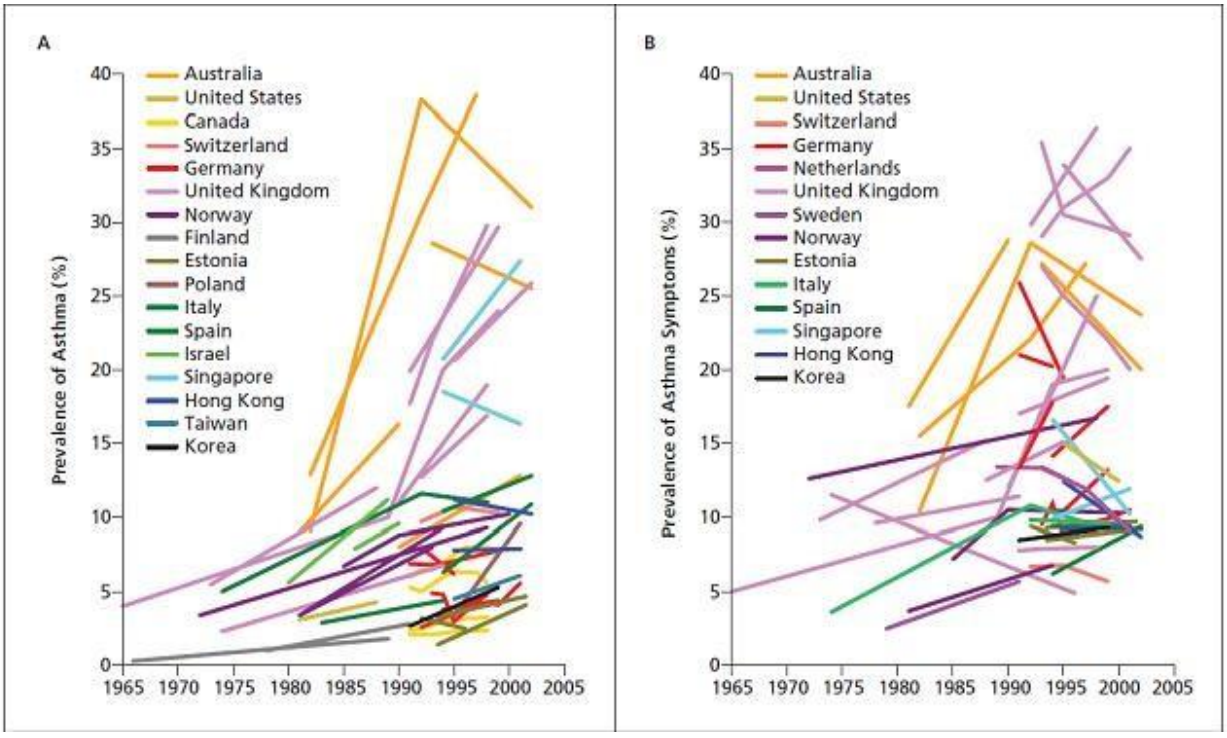
Asthma nearly affects 15 million Americans, in this mostly 5 millions are children. The cases of asthma were increased. The lifestyle and environmental hypothesis showed increase rates of asthma. Death rates are mostly higher among African Americans than among white Americans. It is more common in boys than girls. It is also common in women than men. Between 1960s and 2008, death rates were increased. In most countries, asthma thought to affect 3% of population.

Although genetic predisposition is clearly evident, gene by- environment interaction probably explains much of the international variation in prevalence rates for allergy and asthma. Environmental factors such as infections and exposure to endotoxins may be protective or may act as risk factors, depending in part on the timing of exposure in infancy and childhood. Some prenatal risk factors, including maternal smoking which been firmly established, but diet and nutrition, stress, use of antibiotics and mode of delivery may also affect the early development of allergy and asthma. Later in childhood, putative risk factors include exposure to allergens, breastfeeding (which may initially protect and then increase the risk of sensitization), family size and structure, and sex and gender. In adulthood, recurrence of childhood asthma may be just as common as new-onset asthma.

The examination of epidemiologic risk factors in the development of asthma presented here began in 2004 with a search of MEDLINE, using the Medical Subject Heading (MeSH) terms “asthma,” “longitudinal” and “cohort study.”[10]

Cross-sectional population-based studies such as these are highly dependent on recognition of symptoms, so they do not necessarily reflect the true heterogeneity of asthma. However, a wide variation in prevalence rates has been documented:

studies of both children and adults have revealed low prevalence rates (2%–4%) in Asian countries (especially China and India) and high rates (15%–20%) in the United Kingdom, Canada, Australia, New Zealand and other developed countries.



This figure shows changes in prevalence of diagnosed asthma (A) and asthma symptoms (B) over time among children and young adults[11].

Asthma comprises a range of heterogeneous phenotypes that differ in presentation, etiology and pathophysiology. The risk factors for each recognized phenotype of asthma include genetic, environmental and host factors. Although a family history of asthma is common, it is neither sufficient nor necessary for the development of asthma.

3.4 CLASSIFICATION OF ANTI ASTHMATIC DRUGS[15]

- β – Adrenergic Receptor Agonists
  - Short acting β – Adrenergic Receptor Agonists
    - Albuterol (proventi, ventolin)
    - Levabuterol (xopenex, (R)- enantiomer of albuterol.
    - Metaproterenol (alupent)
    - Terbutaline (brethaire)
    - Pirbuterol (maxair)
  - Long acting β – Adrenergic Receptor Agonists
    - Salmeterol xinafoate (serevent)
  - Oral therapy with β – Adrenergic Receptor Agonists.

- Glucocorticoids
  - Systemic glucocorticoids
  - Inhaled glucocorticoids
    - Beclamethasone dipropionate (beclovent)
    - Triamacinolone acetonide (azmacort)
    - Flunisolide (aerobid)
    - Budenoside (pulmicort)
    - Fluticasone propionate (flovent)

- Leukotriene Receptor antagonists
  - Zafirlukast (accolate)
  - M o n t e l u k a s t (Accolate)

- Leukotriene synthesis inhibitors
  - Zileuton (zyflo)

- Others
    - Cromolyn sodium
    - Nedocromil sodium
    - Theophylline

- Anticholinergic agents
    - Ipratropium bromide (atrovent)

ZAFIRLUKAST

3.5 MECHANISM OF THE ACTION

Cystenyl leukotrienes are important mediators of bronchial asthma.

Zafirlukast is the leukotriene antagonist, which competitively antagonise cysteinyl leukotriene receptor $CysLT_1$ in the lungs which mediates bronchoconstriction, increased vascular permeability and recruitment of eosinophils and results in less inflammation.

Zafirlukast is generally indicated for prophylactic therapy of mild to moderate asthma as alternative to inhaled glucocorticoids.

3.6 PHARMACOKINETICS

The pharmacokinetics of Zafirlukast is as follows:

Absorption

Zafirlukast is rapidly absorbed following oral administration. After administration of the 10-mg film-coated tablet to fasted adults, the mean peak Zafirlukast plasma concentration (Cmax) is achieved in 3 to 4 hours (Tmax). The mean oral bioavailability is 64%. The oral bioavailability and Cmax are not influenced by a standard meal in the morning.

The safety and efficacy of ACCOLATE in patients with asthma were demonstrated in clinical trials in which the 10-mg film-coated tablet and 5-mg chewable tablet formulations were administered in the evening without regard to the time of food ingestion. The safety of ACCOLATE in patients with asthma was also demonstrated in clinical trials in which the 4-mg chewable tablet and 4-mg oral granule formulations were administered in the evening without regard to the time of food ingestion. The safety and efficacy of ACCOLATE in patients with seasonal allergic rhinitis were demonstrated in clinical trials in which the 10-mg film-coated tablet was administered in the morning or evening without regard to the time of food ingestion.

Distribution

Zafirlukast is more than 99% bound to plasma proteins. The steady state volume of distribution of Zafirlukast averages 8 to 11 liters. Studies in rats with radiolabeled Zafirlukast indicate minimal distribution across the blood-brain barrier. In addition, concentrations of radiolabeled material at 24 hours postdose were minimal in all other tissues.

Metabolism

Zafirlukast is extensively metabolized. In studies with therapeutic doses, plasma concentrations of metabolites of Zafirlukast are undetectable at steady state in adults and pediatric patients.

In vitro studies using human liver microsomes indicate that cytochromes P450 3A4 and 2C9 are involved in the metabolism of Zafirlukast. Clinical studies investigating the effect of known inhibitors of cytochromes P450 3A4 (e.g., ketoconazole, erythromycin) or 2C9 (e.g., fluconazole) on Zafirlukast pharmacokinetics have not been conducted. Based on further in vitro results in human liver microsomes, therapeutic plasma concentrations of Zafirlukast do not inhibit cytochromes P450 3A4, 2C9, 1A2, 2A6, 2C19, or 2D6. In vitro studies have shown that Zafirlukast is a potent inhibitor of cytochrome P450 2C8; however, data from a clinical drug-drug interaction study involving Zafirlukast and rosiglitazone (a probe substrate representative of drugs primarily metabolized by CYP2C8) demonstrated that Zafirlukast does not inhibit CYP2C8 in vivo, and therefore is not anticipated to alter the metabolism of drugs metabolized by this enzyme.

Elimination

The plasma clearance of Zafirlukast averages 45 mL/min in healthy adults. Following an oral dose of radiolabeled Zafirlukast, 86% of the radioactivity was recovered in 5-day fecal collections and <0.2% was recovered in urine. Coupled with estimates of Zafirlukast oral bioavailability, this indicates that Zafirlukast and its metabolites are excreted almost exclusively via the bile.

In several studies, the mean plasma half-life of Zafirlukast ranges from 2.7 to 5.5 hours in healthy young adults. The pharmacokinetics of Zafirlukast are nearly linear for oral doses up to 50 mg. There is little accumulation of the parent drug in plasma (14%), during once-daily dosing with 10-mg Zafirlukast.

Drug Interactions

Zafirlukast at a dose of 10 mg once daily dosed to pharmacokinetic steady state:

- did not cause clinically significant changes in the kinetics of a single intravenous dose of theophylline (predominantly a cytochrome P450 1A2 substrate).

- did not change the pharmacokinetic profile of warfarin (primarily a substrate of CYP 2C9, 3A4 and 1A2) or influence the effect of a single 30-mg oral dose of warfarin on prothrombin time or the INR (International Normalized Ratio).
- did not change the pharmacokinetic profile or urinary excretion of immunoreactive digoxin.
- did not change the plasma concentration profile of terfenadine (a substrate of CYP 3A4) or fexofenadine, its carboxylated metabolite, and did not prolong the QTc interval following co-administration with terfenadine 60 mg twice daily.

Indications and usage

ACCOLATE is indicated for the

- prophylaxis and chronic treatment of asthma in adults and pediatric patients 12 months of age and older.
- prevention of exercise-induced bronchoconstriction in patients 15 years of age and older.
- relief of allergic rhinitis (seasonal allergic rhinitis in adults and pediatric patients 2 years of age and older, and perennial allergic rhinitis in adults and pediatric patients (6 months of age and older).

CONTRAINDICATIONS

Hypersensitivity[13].

PLAN OF WORK

STUDY OBJECTIVES

The basic aim of this project was to study the conductance of BE study, in accordance with the regulatory guidelines. The BE study was conducted on a test product, Zafirlukast and a reference product singuliar.

The study objectives included:

i. Assessment of the bioavailability of test product A while comparing with a reference product B in 12 healthy, normal, adult, human subjects under fasting conditions.
ii. Investigate the source of the observed variability in the C_{max} of Test drug.

DESIGN AND CONDUCT OF STUDY

A bioequivalence study is basically a comparative bioavailability study designed to establish equivalence between test and reference products.

The design should be based on a reasonable knowledge of the pharmacodynamics and/or the pharmacokinetics of the active substance in question. The design and conduct of the study should follow ICH/ EU-regulations on Good Clinical Practice, including reference to an Ethics Committee. The rights, safety, and well being of all trial subjects must always be respected and should be given special attention.

Study Design

The study should be designed in such a way that the formulation effect can be distinguished from other effects. If the number of formulations to be compared is two, a two-period, two-sequence crossover design is often considered to be the design of choice.

However, under certain circumstances and provided the study design and the statistical analyses are scientifically sound alternative well-established designs could be considered such as parallel design for very long half-life substances and replicate designs for substances with highly variable disposition

In general, single dose studies will suffice, but there are situations in which steady-state studies may be required, e.g. in the case of

- dose- or time-dependent pharmacokinetics,
- some modified release products (in addition to single dose investigations),

or can be considered,

e.g.

- if problems of sensitivity preclude sufficiently precise plasma concentration measurements after single dose administration.

- if the intra-individual variability in the plasma concentration or disposition precludes the possibility of demonstrating bioequivalence in a reasonably sized single dose study and this variability is reduced at steady state.

For several drugs a great inter-subject variability in clearance is observed. The intra-subject coefficient of variation (approximately 15%) is usually substantially smaller than that between subjects (approximately 30%), and therefore, crossover designs are generally recommended for bioequivalence studies.

The primary advantage of the crossover design is that since the treatments are compared on the same subject, the intersubject variability does not contribute to the error variability.

Inherent in both the crossover and parallel designs are the two fundamental statistical concepts of study design, namely

1. Randomization,
2. Replication and Error control.

Randomization implies allocation of treatments to the subjects without selection bias. Consequently, randomization is essential to determine an unbiased estimate of the treatment effects.

In the present study the randomization schedule was generated using the PROC PLAN procedure on statistical package SAS, version 9.1. All the subjects were randomly assigned either of the two treatment sequences i.e. “AB” or “BA”. The randomization schedule was balanced over the period and sequence and all the subjects were dosed, in each period, as per the randomization schedule.

Replication implies that a treatment is applied to more than one experimental unit (subject) to obtain more reliable estimates than is possible from a single observation and hence provides a more precise measurement of treatment effects. The number of replicates (sample size) required depends upon the degree of differences to be detected and inherent variability of the data.

Replication is used concomitantly with "Error control" to reduce the experimental error or error variability.

In the present study the design followed was single dose, open-label, analyst-blind, two- treatment, two-period, two-sequence, crossover bioequivalence study.

It is an open labeled study as the subjects and the investigator were not be blinded towards the identity of the study medications. Only the analysts were blinded towards identity of study medication administered.

Washout Period

Subsequent treatments should be separated by periods long enough to eliminate the previous dose before the next one (adequate wash out periods). In steady-state studies wash out of the previous treatment last dose can overlap with the build-up of the second treatment, provided the build-up period is sufficiently long (at least three times the terminal half-life).

In the present study drug administration in first period was followed by a washout period of at least 7 days before subjects were switched over to the other treatment in the second period.

Selection of subjects:

The subject population for bioequivalence studies should be selected with the aim to minimise variability and permit detection of differences between pharmaceutical products. Therefore, the studies should normally be performed with healthy volunteers. The inclusion/exclusion criteria should be clearly stated in the protocol. Subjects could belong to either sex; however, the risk to women of childbearing potential should be considered on an individual basis.

In general, subjects should be between 18 - 55 years old capable of giving informed consent and of weight within the normal range according to accepted normal values for the Body Mass Index (BMI) of 18-25. The BMI is calculated using the formula:

$$BMI = \frac{\text{Weight in Kgs}}{\text{Height in m}^2}$$

The number of subjects required is determined by

- the error variance associated with the primary characteristic to be studied as estimated from a pilot experiment, from previous studies or from published data,
- the significance level desired,
- the expected deviation from the reference product compatible with bioequivalence (delta, i.e. percentage difference from 100 %)and
- the required power.

The number of subjects required is calculated by the formula:

$$\mathbf{N=(t_{\alpha,\,2N-2}+t_{\beta,\,2N-2})^2\,[CV/(V-\delta)]^2}$$

Where N=number of subjects

T=appropriate value from the t-distribution

α=type 1 error

β=type 2 error

δ=Treatment difference

CV=coefficient of variance (intra subject)

V=Bioequivalence limit

Calculations are quite tedious and time consuming, thus it is done using statistical software and statistical tools[14].

Pharmacokinetic Sampling

Under normal circumstances, blood, rather than urine or tissue, should be used. In most cases, drug, or metabolites are measured in serum or plasma. However, in certain cases whole blood may be more appropriate for analysis.

Blood samples should be drawn at appropriate times to describe the absorption, distribution, and elimination phases of the drug. The sampling schedule should be planned to provide an adequate estimation of Cmax and to cover the plasma concentration time curve long enough to provide a reliable estimate of the extent of absorption. This is generally achieved if the AUC derived from measurements is at least 80% of the AUC extrapolated to infinity. For most drugs, 12 to 18 samples, including a predose sample, should be collected per subject per dose. This sampling should continue for at least three or more terminal half lives of the drug. The exact timing for sample collection depends on the nature of the drug and the input from the administered dosage form. The sample collection should be spaced in such a way that the maximum concentration of the drug in the blood (Cmax) and terminal elimination rate constant (λ_z) can be estimated accurately.

For drugs with a long half-life, relative bioavailability can be adequately estimated using truncated AUC as long as the total collection period is justified.

According to the C_{max} and T_{max} values of drug XY, the sampling schedule and amount of blood to be collected was decided.

In each period, a total of 18 venous blood samples were collected from each subject as per the following schedule:

Predose (00 hr), 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 6.00, 8.00, 10.00, 12.00, 16.00, 20.00, 24.00 hrs post dose in each period.

The total volume of blood collected from each subject during the study will be 250ml for males and 258ml for females as follows:

- Pre dose and post-dose samples (36 samples of 06 mL each) = 216 mL
- Discarding of saline mixed blood samples resulting from use of intravenous cannula (0.5 mL each time) = 18 mL
- Screening and post study laboratory assessments = 16 mL
- Serum pregnancy test (if female) = 8 mL

DESIGN OF BA/BE FACILITY

A general BE study facility includes various departments like the Clinical department, Bioanalytical department, Bio-statistics and Data management division and a Quality assurance (QA) department. Each department in turn consists of different functional areas.

The Clinical facility has many subdivisions

- Clinical Pharmacological Units (CPU) with areas for Phlebotomy, Dosing stations, recreation and refreshment rooms with safety precautions taken.
- Separate areas for Pharmacy, sample separation, deep freezers
- Full time medical vigilance and care
- ICU and emergency medical care services
- Diagnostic lab services
- Biowaste disposal services

The Bioanalytical facility comprises of various functional areas like:

- Analytical lab
- Sample processing room
- Washing room

- Scientist room
- Store room for chemicals and solvents
- Mass balance room

The Biostatistics and Data management division is associated with functions like:

- Sample size calculations
- Statistical analysis
- Study design
- Data collection, verification and analysis
- Report preparation as per the regulatory standards

Quality Assurance department generally functions as an independent unit for indirect enforcement of stringent quality standards to the whole study process and system. It is responsible for planning and conducting regular audits in all the departments to ensure that all the activities are carried out in accordance to the approved protocol, and in accordance with the laboratory standards and regulatory. It independently verifies all the raw data generated during the study process for its completeness, accuracy and authenticity.

CHARACTERISTICS TO BE INVESTIGATED

In most cases evaluation of bioavailability and bioequivalence will be based upon the measured concentrations of the parent compound. In some situations, however, measurements of an active or inactive metabolite may be necessary instead of the parent compound. The use of a metabolite may be advantageous to determine the extent of drug input, e.g. if the concentration of the active substance is too low to be accurately measured in the biological matrix (e.g. major difficulty in analytical method, product unstable in the biological matrix or half-life of the parent compound too short) thus giving rise to significant variability.

In bioavailability studies, the shape of and the area under the plasma concentration versus time curves are mostly used to assess extent and rate of absorption. The use of urine excretion data may be advantageous in determining the extent of drug input in case of products predominately

excreted renally. From the primary results, the bioavailability characteristics desired are estimated, namely AUCt, AUC∞ , Cmax, Tmax, Aet, Ae∞ as appropriate, or any other justifiable characteristics. In bioequivalence studies the AUCt is the most reliable reflection of the extent of absorption[15].

The following pharmacokinetic parameters are required for submission:

- Plasma concentrations and time points
- Subject, period, sequence, treatment
- AUC0-t, AUC0-∞, Cmax, Tmax,λz , and t1/2.
- Intersubject, intrasubject, and/or total variability, if available
- Cmin (concentration at the end of a dosing interval),
- Cav (average concentration during a dosing interval),
- Degree of fluctuation [(Cmax-Cmin)/Cav]

The following statistical information required for AUC0-t, AUC0-∞, and Cmax:

- Geometric mean
- Arithmetic mean
- Ratio of means
- Confidence intervals

Rounding off of confidence interval values:

Confidence interval (CI) values should not be rounded off; therefore, to pass a CI limit of 80-125, the value should be at least 80.00 and not more than 125.00.

CLINICAL COMPONENT

Communication from Sponsor

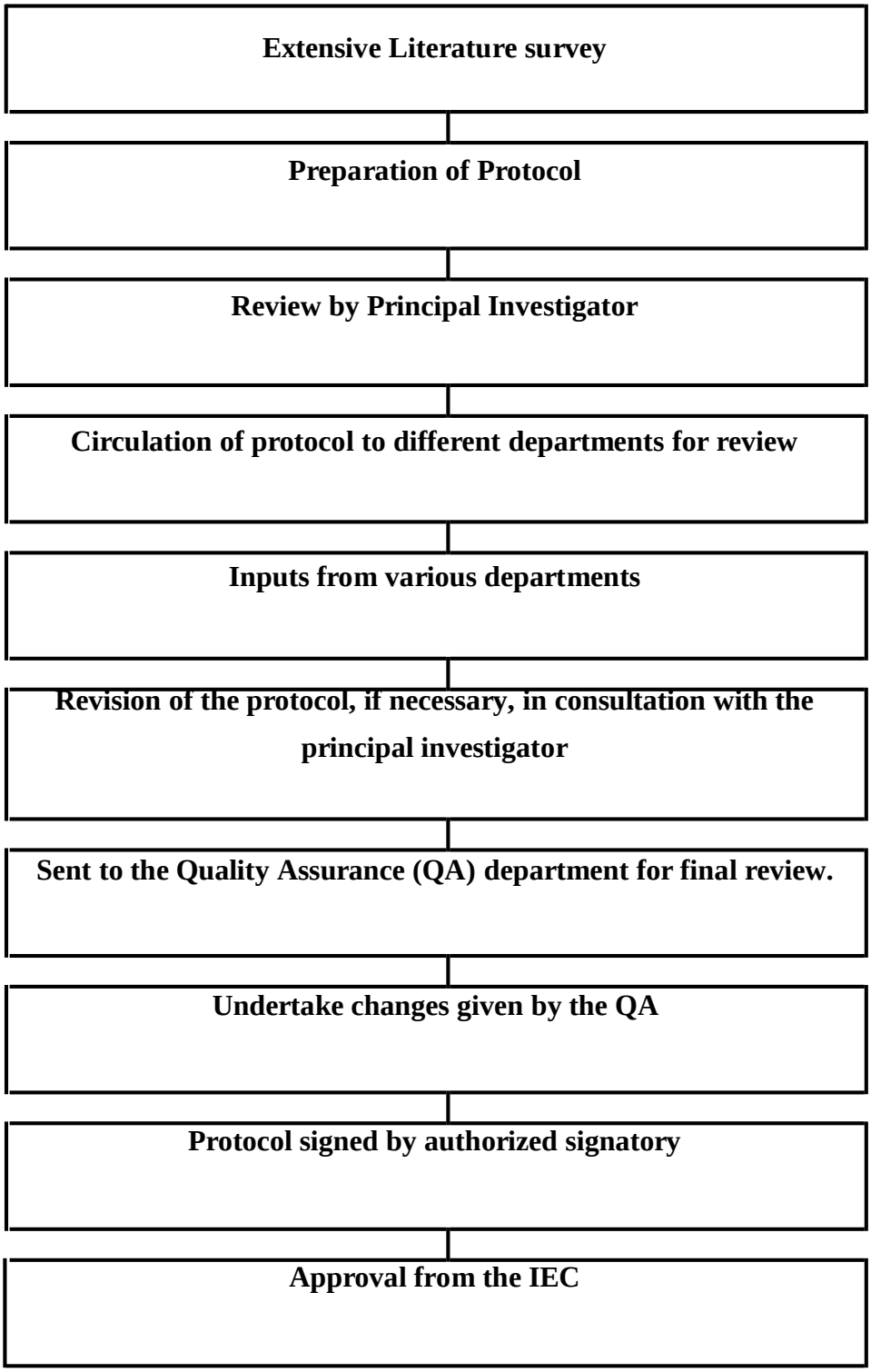
Sponsor is an individual, company, institution or organization which takes responsibility for the initiation, management, and financing of a clinical study for the veterinary product under investigation. The investigator of the study, after receiving information from the sponsor, starts preparing the protocol according to which the study is conducted.

Preparation of Protocol

Protocol is defined as a document signed and dated by the investigator and the sponsor that fully describes the objective(s), design, methodology, statistical considerations and organization of a study. The study protocol may also give the background and rationale for the study but these could be provided in other study protocol-referenced documents.

The protocol includes all the details regarding the investigational product, the details regarding the administration of the drug, Pharmacokinetic (PK) sample withdrawal time-points, safety assessment parameters etc.

A protocol is prepared by the investigators of the study or his designee and reviewed by various departments like analytical, statistical, QA to make necessary changes. The following chart gives an overview regarding the preparation of the protocol.


Extensive Literature survey
Preparation of Protocol
Review by Principal Investigator
Circulation of protocol to different departments for review
Inputs from various departments
Revision of the protocol, if necessary, in consultation with the principal investigator
Sent to the Quality Assurance (QA) department for final review.
Undertake changes given by the QA
Protocol signed by authorized signatory
Approval from the IEC

Protocol summary:

Study title:	An open label, randomized, two-period, two-sequence, single dose Crossover comparative oral bioavailability study of Zafirlukast tablet 20 mg (test) of aurobindo pharma ltd and Accolate tablets 20 mg (reference) of Merck sharp & Dohme ltd, UK in 12 healthy adult, human subjects under fasting conditions.
Study objectives:	To compare the rate and extent of absorption of Zafirlukast tablets 10 mg (test) of aurobindo pharma ltd, india with that of singuliar tablets 10 mg (reference) of merck sharp and dohme ltd, UK. To monitor adverse events and to ensure the safety of subjects.
Study design:	An open label, randomized, two- treatment, two sequence, two Period , single dose, comparative oral bioavailability study in 12 healthy, adult, human subjects under fasting conditions.
Sample size:	12 healthy, adult human subjects.
Study treatments:	Reference : Accolate 20 mg Test: Zafirlukast tablets 20 mg.
Screening:	Healthy volunteers aged from 18 years or older with body mass index (BMI) between 18.5- 30 kg/m^2 of either sex males

	or females will be selected according to inclusion and exclusion criteria.
Dose:	A single oral dose of Zafirlukast tablets 20 mg of reference and test product will be dosed with atleast 150 ml of drinking water under fasting conditions.
Sampling schedule:	At Predose (00 hr), 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 6.00, 8.00, 10.00, 12.00, 16.00, 20.00, 24.00 hrs post dose in each period.
Blood loss:	The total volume of blood withdrawn for each subject will be 250 for males and 258 for females.
Housing:	Atleast 11 hours before dosing and until 30 hours post dose in each period.
Wash out period:	Atleast 5 days between each treatment schedule.
Analyte:	Zafirlukast will be estimated in plasma using a validated method.
PK parameters & analysis :	T_{max}, C_{max}, AUC_{0-t}, $AUC_{0-\alpha}$, $AUC_{\% \text{ extrapolation}}$, K_{el} and $T_{1/2}$ will be determined from Zafirlukast data using WIN NONLIN software.
Statistical analysis:	Summary statistics, ANOVA, 90% confidence interval, ratio analysis, intrasubject variability and power will be caluclated for Zafirlukast pharmacokinetic data using SAS^R

version 9.1.3 at pharmacokinetics and biostatistics unit of APL research centre, Hyderabad, India.

Bioequivalence criteria: The test product is considered as bioequivalent to the reference product, if 90% confidence interval for ratio of population geometric means of Ln-transformed parameters $C_{max,}$ AUC_{0-t} and $AUC_{0-\alpha}$ of Zafirlukast is within the acceptence interval of 80.00% - 125.00%.

INDEPENDENT ETHICS COMMITTEE

Independent Ethics Committee (IEC) consists of a board of members who look into the ethical issues of the study to be conducted. The study operations can be initiated only after the protocol is approved by IEC.

An IEC should safeguard the rights, safety, and well being of all trial subjects. Special attention should be paid to trials that may include vulnerable subjects. The investigator may provide information on any aspect of the trial, but should not participate in the deliberations of the IEC or in the vote/opinion of the IEC. An IEC may invite nonmembers with expertise in special areas for assistance.

Composition, Functions, and Operations

The IEC consists of a reasonable number of members, who collectively have the qualifications and experience to review and evaluate the science, medical aspects, and ethics of the proposed trial. It is recommended that the IEC should include: at least five members.

(b) At least one member whose primary area of interest is in a nonscientific area.

(c) At least one member who is independent of the institution/trial site.

- Only those IEC members who are independent of the investigator and the sponsor of the trial should vote/provide opinion on a trial-related matter.
- A list of IEC members and their qualifications should be maintained.
- It should perform its functions according to written operating procedures, should maintain written records of its activities and minutes of its meetings, and should comply with GCP and with the applicable regulatory requirement(s).
- Only members who participate in the IEC review and discussion should vote/provide their opinion and/or advise.

Protocol Training

After the approval of the protocol, it is discussed among the investigators of the study. The summary of the protocol that includes:

- The name of the investigational product (drug to be administered to the subjects), Reference drug
- Dose to be administered
- Type of study whether it is a single center study, a fast or fed study, analyst study etc.
- Number of subjects to be enrolled in the study
- Kind of study etc, and other minute details like the Clinical Pharmacology unit (CPU) in which the subjects would be housed etc,

This summarized version of the protocol is discussed among the personnel in the facility to train them in the protocol.

Registration of Volunteers

For recruiting volunteers for a study suitable volunteers are selected from the database. New people are informed and registered in the database after the consent. Generally, healthy volunteers in the age group of 18-45 years are preferred based on inclusion and exclusion criteria[14].

Inclusion criteria

Subjects must fulfill the following criteria to be considered for inclusion into this study.

- Healthy volunteers within age range of 18- 50 yrs.
- A body mass index of 18-25 kg/m^2.
- Given written informed consent to participate in the study.
- Absence of disease markers of HIV 1 & 2, hepatitis B & C virus & RPR.
- Absence of significant disease or clinically significant abnormal laboratory values on laboratory evaluation, medical history and physical examination during the screening.
- A normal 12- lead ECG.
- A normal chest X-ray (PA view)
- Compliance with the requirement of the entire protocol.
- No history or no evidence of hypersensitivity to Zafirlukast and its formulation components.
- No history of gastrointestinal problems (ulcers)
- No history of significant systemic disorders.
- No history of psychiatric disorders.
- No history of allergic rash.
- No donation of blood (one unit or 350 ml) within 56 days prior to study check-in.
- No history of addiction to any recreational drug or drug dependence.
- No participation in any recreational drug or drug dependence.
- No participation in any clinical study within past 56 days.

- No receipt of any prescription drugs or over the counter drugs (eg. Cold preparations, antacid precipitations, vitamins and natural products used for therapeutic benefits) within two weeks prior to study check-in.
- No history of dehydration from diarrhea, vomiting or any other reason with- in period of 24 hrs prior to study check-in.
- No family history of neurological disorders.
- Not consumed alcohol and xanthine containing food & beverages (chocolates, tea, coffee or cola drinks) cigarettes & tobacco products, for atleast 48 hrs prior to study check-in.
- Not consumed grape fruit (mosumbi/ sweet lime) juice within 48 hrs prior to study check in
- Negative results for drugs of abuse (benzodiazepines, cocaines, opioids, amphetamines, cannabinoids and barbiturates) in urine during the study check-in of each period.
- Female volunteer demonstrating a negative pregnancy test.
- If study volunteer is female or is of child bearing potential practicing an acceptable method of birth control for the duration of study as judged by the investigator(s), such as condoms, jellies, foams, diaphragm, intrauterine devices(IUD), or abstinence.

  (Or)

  Is postmenopausal for atleast 1 year.

  (Or)

  Is surgically sterile (bilateral tubal ligation, bilateral oophorectomy or hysterectomy has been performed on the study volunteer)

Exclusion criteria

The subjects will be excluded based on the following criteria

- History of seizures.
- History of alcohol consumption for more than two units / day (1 unit = 30 ml of spirit or 1 pint of beer) or having consumed alcohol within 48 hrs prior to check-in.
- High caffeine (more than 5 cups of coffee or tea / day) or tobacco (more than 9 cigarettes / beedies / cigars per day) consumption.
- History of difficulty in donating blood or difficulty in accessibility of veins.

- An unusual or abnormal diet for whatever reason eg. Because of fasting due to religious reasons.
- Received pharmacological agents known to significantly induce or inhibit drug metabolizing enzymes within 14 days of the start of the study.
- Female volunteers who are currently breast feeding.
- Female volunteers who has used implanted or injected hormonal contraceptives anytime during the 6 months prior to study or used hormonal contraceptives within 14 days before dosing.

Withdrawal criteria

Subjects may be withdrawn from the study by the principal investigator/ clinical investigator/ attending physician for any of the following reasons during the course of the study.

- If the subject suffers from significant illness.
- After dosing, if the subject vomits, at or before 2 times the median T_{max} of Zafirlukast in any period.
- If the subject requires unacceptable concomitant medications.
- If the subject has entered the study in violation of the exclusion and inclusion criteria.
- If the subject found to be non co-operative.
- If the subject decides to voluntarily withdraw from the study.

  Note:

  Any subject withdrawals will be reported for;

  - Reasons for withdrawal (if any)
  - Clinical assessment of the subject will be done at the time of withdrawal if subject withdraws during the residential stay.

Screening

The registered individuals are screened, by taking their consent. Screening generally includes:

- Body mass index (BMI)

- ECG
- Medical examination that involves general & systemic examination
- Chest X-ray
- Laboratory tests including Hemogram, liver function test (LFT), renal function test (RFT), Serology & other details.
- Urine test to determine the consumption of drugs like morphine, codeine, LSD or any other drug of abuse (on the day of check in)
- Breath alcohol test (on the day of check in)

Doctors along with nurses and phlebotomists present in the facility would be screening the volunteers. The volunteers are given a particular date to report to the facility and participate in the informed consent session prior to enrollment in the study.

Informed Consent Form (ICF)

Informed Consent is a documented process by which an owner, or owner's agent, voluntarily confirms the owner's willingness to allow their animal(s) to participate in a particular study, after having been informed of all aspects of the study that are relevant to the decision to participate.

ICF is designed as per the ICH-GCP and local regulatory requirements. ICF is conducted in order to get the consent from the volunteer to participate in the study. It is conducted for enrollment and general screening of a volunteer. He would be given all the information regarding the study including:

- Details of Investigational products
- Adverse events that may occur during the study
- The total blood loss
- The compensation to be given at the end of the study
- Regulations to be followed while participating in the study

He would be given the freedom to withdraw from the study at any point of time, during the study. This consent is taken as a part of the ethical issue in conducting a BA/BE study. Every care is taken to protect the health of the volunteer. The volunteers would sign on this form and

give their consent for participating in the study. Once they enroll in to the study, they would be called 'subjects'. The enrollment in the study starts in the "check-in" process.

Check-in Process

The volunteers who gave in their consent to participate in the study would be enrolled in the study i.e. the check-in process. During this process it is checked whether the person has met all the inclusion/exclusion criteria and cleared the screening process. They will undergo vital examination and Medical examination again to ensure they are fit for participation in the study. They would be changing into the uniforms provided to them in the facility. Once the check-in of the volunteer is completed he would be called as 'subject'. The subjects are provided with all the requirements they need including recreational activities like movies and games, newspapers. The check in day is called as Day 0.The subjects are given standardized dinner after which they will be fasting overnight for 10 hours.

Dosing of Investigational product

The subjects are dosed next morning with the investigational product (IP) in the study after they have maintained 10hr fasting as per protocol. The dosing day is called as Day 1. Dosing is done according to the procedure mentioned in the protocol. The randomization code for the dosing is generated by the statistician, in which the sequence of IP administration is mentioned (eg. AB, BA). The IP is administered in presence of principal investigator. It is observed that the subject takes the IP accordingly with required amount of water and swallows it completely. Once the dosing is completed the blood samples (PK samples) from the subject would be collected at time intervals mentioned in the protocol. In fed studies high fat breakfast is provided to the subjects 30 minutes prior to dosing. One hour predose and 2 hours post dose water restriction was maintained.

Collection of PK samples

The subjects are cannulated on the day of dosing so that the blood withdrawal would be easier and to avoid the repeated needle pricks. Fixed volume of blood (generally 6 ml) is withdrawn at each time point. This is collected in the vacutainers containing the adequate amount of anticoagulant as per described in the protocol (generally K3 EDTA). To avoid the mixing of the

blood with the residual blood in the cannula 0.5ml blood is discarded before every sample collection. After the sample is collected 0.5ml saline will be pushed in the cannula to avoid the blockage of the cannula.

The collected blood samples are sent to the separation room where the plasma is separated, after centrifugation at defined parameters in the protocol (generally 3000 rpm for 10 minutes)

Safety assessment

To ensure the well being of the subject after the administration of IP, vital signs of the subjects are checked at regular intervals of time defined in the protocol. If there is any complaint form the subject the Medical officer looks after it and document it as an Adverse event and follow it up till the resolution. The normal vital signs range is as follows:

Temperature	97.8 °F-99 °F
Pulse Rate	60-100 beats/min
Respiration Rate	14-20/min
Systolic BP	100-138mm of Hg
Diastolic BP	60-88mm of Hg

Check out process

After the completion of the study the subjects are checked- out. In the check out process the subjects undergo a medical check up to ensure that they are healthy even after participating in the study. The study cycle is repeated after the washout period when the subjects are crossed over to other treatment. Their post study medical check up includes the blood test. In case of long acting drugs the subjects are required to come for ambulatory samples after being checked out of the

facility. Once the subjects finish giving their PK samples they are paid their compensation as per the protocol.

Sample separation and Storage

The collected PK samples received in vacuettes would be centrifuged at a particular RPM (3000 rpm) at a temperature of 4 °C for around 10-12 minutes. The plasma gets separated due to the centrifugal force. This separated plasma is collected in two different tubes called aliquots. The aliquots are pre-labeled and sent to the deep freezer where the samples are stored at a temperature of –60 °C to –70 °C.

Various precautions are taken while separating the plasma. The plasma is separated without touching the bed of blood cells. Care should be taken that the samples are not exposed to room temperature for a long time.

Every detail regarding the receival of the sample and reporting of hemolysed samples is documented in various forms.

Sample Sorting

Once all the samples from the subjects are collected, including the ambulatory samples, the samples are sorted. The sorting is done by separating the aliquots containing samples of different time points of each subject into easy sealing bags. Various conditions are maintained while sorting the samples, like the maintenance of low temperatures. Sorting is done in presence of dry ice to prevent the exposure of samples to room temperature and also to prevent their degradation due to thawing.

These bags are sealed into various boxes and stored in the deep freezer, which are later handed over to the bio-analytical department with proper documentation for further processing.

BIOANALYSIS

The bioanalytical part of bioequivalence trials should be conducted according to the applicable principles of Good Laboratory Practice (GLP). (EMEA / OECD GLP / WHO GLP STANDARD). The bioanalytical methods used to determine the active moiety and/or its biotransformation product(s) in plasma, serum, blood or urine or any other suitable matrix must be well characterized, fully validated and documented to yield reliable results that can be satisfactorily interpreted.

Materials and Methods

The list of materials and methods used during analytical phase of the study are discussed below:

Materials

Table No. 1: Investigational Products:

Product	Dose
Test product A: Zafirlukast	20 mg
Reference product B: Accolate	20 mg

Table No. 2: Reference Standards:

The reference standards of drug Zafirlukast and internal standard IS were procured.

Compound	Use
Zafirlukast	Analyte
IS	Internal Standard

Reagents and Chemicals

Acetonitrile, Formic acid, Ammonium acetate, Methanol (HPLC Grade) and Milli-Q Water.

Instrumentation

Shimadzu HPLC equipped with pump, auto sampler, Mass spectrometer MDS SCIEX API 4000 LC/MS/MS and data acquisition system (Analyst Software Version 1.4.1) were used for the quantitative determination of analyte in human plasma.

Method

Method development

The method development and establishment phase defines the chemical assay. Before beginning of the analysis for a molecule a method for its analysis has to be developed. The physico-chemical properties of the analyte play an important role in deciding various factors during the method development. The volume of reagents to be used, mobile phase compositions, extraction

techniques, various spectroscopic parameters like the flow rate, split ratio, voltage, gas parameters, parent and daughter ion masses etc are decided during the development phase.

While developing an analytical method for drug Zafirlukast, it was found that the solid phase extraction technique was found to give better results during analysis. The resolution of the analyte peak and the peak shape were found to be good when 2mM Ammonium acetate and acetonitrile was used. C-18 cyano column was used. The masses of the parent and daughter ion were set during the tuning phase while setting the various potential and gas parameters.

Method Validation

The main objective of method validation is to demonstrate the reliability of a particular method for the quantitative determination of an analyte(s) concentration in a specific biological matrix.

The fundamental parameters for a bioanalytical method validation are:

- Accuracy,
- Precision,
- Selectivity,
- Sensitivity,
- Reproducibility,
- Stability.

In addition, the stability of the analyte in spiked samples should be determined. Typical method development and establishment for a bioanalytical method include determination of

- selectivity,
- accuracy, precision, recovery,
- calibration curve,
- stability of analyte in spiked samples.

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. For selectivity, analyses of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from at least six sources. Each blank sample should be tested for interference, and selectivity should be ensured at the lower limit of quantification (LLOQ).

Accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte.

Acceptance criteria: The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%.

Precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix.

Acceptance criteria: The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV.

Recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery experiment was performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery.

Biological Matrix

Human plasma containing Tri-potassium Ethylene Diamine Tetra Acetic acid (K_3EDTA) as anticoagulant was procured from blood bank and chromatographically screened for interfering peaks prior to use. The batches free from endogenous interferences were pooled for bulk spiking

of calibration curve (CC) standards and the quality control (QC) samples. During subject sample analysis, a pre-dose sample and pre-dose with IS of each subject in each period was used to check for interference from contaminants or endogenous compounds.

Stock Solutions and Dilution Preparations

The stock solution of drug Zafirlukast was prepared in a volumetric flask by dissolving the drug in methanol and final volume was made up using the same. The stock solution was diluted using a mixture of methanol and milli-Q-water to prepare calibration curve standards for spiking in plasma. The stock dilutions for quality control samples were prepared by diluting the stock solution of drug Zafirlukast sodium using Methanol and Milli-Q water mixture for spiking in plasma. The stock solutions were stored between normal refrigerator temperatures.

The stock solutions for IS (three different concentrations) were prepared in a volumetric flask, using methanol. Internal standard dilutions (three different concentrations) were prepared by diluting the above stocks with methanol and Milli-Q water mixture. The stock solutions of internal standard were stored between normal refrigerator temperatures.

Preparation of Calibration Curve Standards

To the screened human blank plasma, solutions of calibration standards (A, A1, B, C, D, E, F, G, H, and H1) of the drug Zafirlukast were added and the volume was made up with the same blank plasma. Aliquots were prepared using fixed volume of each standard and stored below $-50^{o}C$.

Preparation of Quality Control Samples

The QC samples (LQC, MQC and HQC) were prepared by spiking drug Zafirlukast solutions to the screened human blank plasma and the volume was made up with the same blank plasma. Aliquots were prepared using fixed volume of each standard and stored below –50^{o}C.

Chromatographic Conditions

Column	C-18 Cyano column
Mobile Phase	Acetonitrile: 2mM Ammonium acetate
Rinsing solution	Acetonitrile: Milli-Q water
Retention time	Drug : 1.80 min – 2.20 min and IS: 1.80 min – 2.20 min

Mass Spectrometric Parameters:

Mass spectrometer	API 4000 LC/MS/MS
Ion source	Electron spray ionization
Polarity	Positive
Mass Transitions (Parent / Fragment) m/z	
Zafirlukast	$M_1 \rightarrow m_1$
IS	$M_2, \rightarrow m_2$

Sample Processing & Extraction Technique

Spiked plasma samples were retrieved from the deep-freezer and thawed in a water bath maintained at room temperature. The thawed samples were vortexed to ensure complete mixing of the contents. A fixed volume of IS dilution was added to prelabelled test tubes except in blank samples. To these test tubes the spiked plasma sample was added and vortexed. This was followed by addition of 0.2% formic acid buffer and vortexed again. These samples were then extracted using the solid phase extraction technique.

In solid phase extraction technique, SPE cartridge of 1 cc (30 mg) was used. The cartridges were conditioned on a Speed-disk® pressure processor. using 1 mL each of methanol and Milli-Q water under constant positive pressure of 15 psi. Prepared plasma samples were loaded and followed by washing with Milli-Q water. The cartridges were dried under positive pressure and eluted with mobile phase. The eluent was centrifuged at 3220 ± 50 RPM at 5°C for 5 minutes. The clear supernatant was taken into vials and the samples were analyzed on LC/MS/MS.

Subject sample analyses

Subject Samples were processed along with QC samples (LQC, MQC and HQC). During subject sample analysis, with each CC, samples of two subjects were analyzed in 8 batches and samples of single subject were analyzed in 10 batches.

Identified repeats were analyzed in 2 batches. In all the cases, batch was rejected or accepted based on the results obtained for QCs run with that particular batch The subject samples were analyzed within 3 freeze thaw cycles[17].

Data Collection

All data collection and integration (area mode) were performed by Analyst version 1.4.1 software systems. The intercepts and goodness of fit were determined by least squares linear regression analyses using the ratios of drug / internal standard peak areas of the CC standards. The concentrations of all the study samples, CC standards and QC samples were calculated by Analyst version 1.4.1 software systems. A weighting factor of $1/(\text{concentration})^2$ was used in the calculation of linear regression line with the following equation.

$$\mathbf{y = mx + b}$$

Where, y = peak area ratio of analyte / internal standard

m = slope of the CC

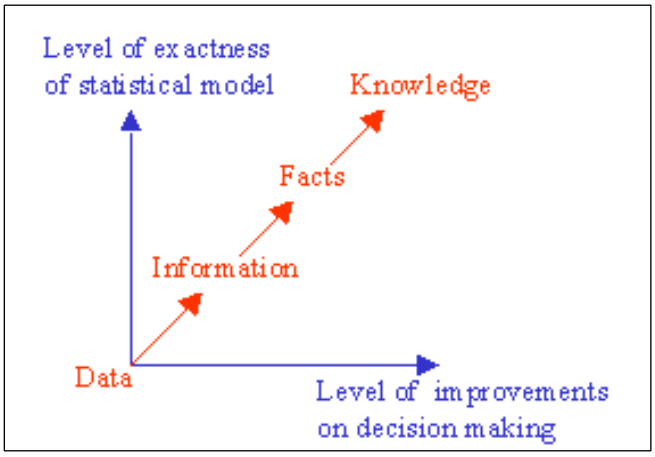
x = concentration ratio of analyte / internal standard

b = y-axis intercept of the CC

Statistical Interpretation

There is a growing need of the application of mathematical statistics to a wide range of biological processes. Most people working in scientific research are forced to apply some concepts of biostatistics into their work if they want to share their results with the scientific community. This community needs to be convinced in order to trust your approach. And this is mostly done by biostatistics in forms of significance tests, confidence intervals and so on. A researcher is forced to show that his experimental outcome is not just a matter of chance for convincing other researcher to apply his methodology.

The following figure illustrates the statistical thinking process based on data in constructing statistical models for decision-making under uncertainties.



The above figure depicts the fact that as the exactness of a statistical model increases, the level of improvements in decision-making increases. That's why we need statistical data analysis.

The post study data obtained is analyzed using various statistical tools like the t-test, ANNOVA and the software like SAS and WinNonlin.

Analysis Of Variance (ANOVA), a calculation procedure to allocate the amount of variation in a process and determine if it is significant or is caused by random noise. Using this statistical tool different parameters like the formulation effect, sequence affect, treatment effect between the test product and the reference product can be analysed. Based on the results obtained in this test the bioequivalence between the two products can be determined.

WinNonlin® is the industry standard for pharmacokinetic, pharmacodynamic, and noncompartmental analysis. In addition to its extensive library of built-in PK, PD and PK/PD models, WinNonlin supports custom, user-defined models to address any kind of data.

WinNonlin provides a complete solution with data management, statistical, modeling, and visualization tools in one package. Its worksheet interface facilitates data handling and transformations. Its descriptive statistics and linear mixed effects modeling engines provide flexible pre- and post-modeling analyses. The bioequivalence wizard supports FDA standards for average, individual and population bioequivalence assessment. Additional tools enable exploration of your drug's properties through non-parametric superposition, semicompartmental modeling, deconvolution and nonparametric analysis of crossover studies. Finally, WinNonlin chart and table wizards, and automatic chart output from modeling, produce high-quality output for your study reports.

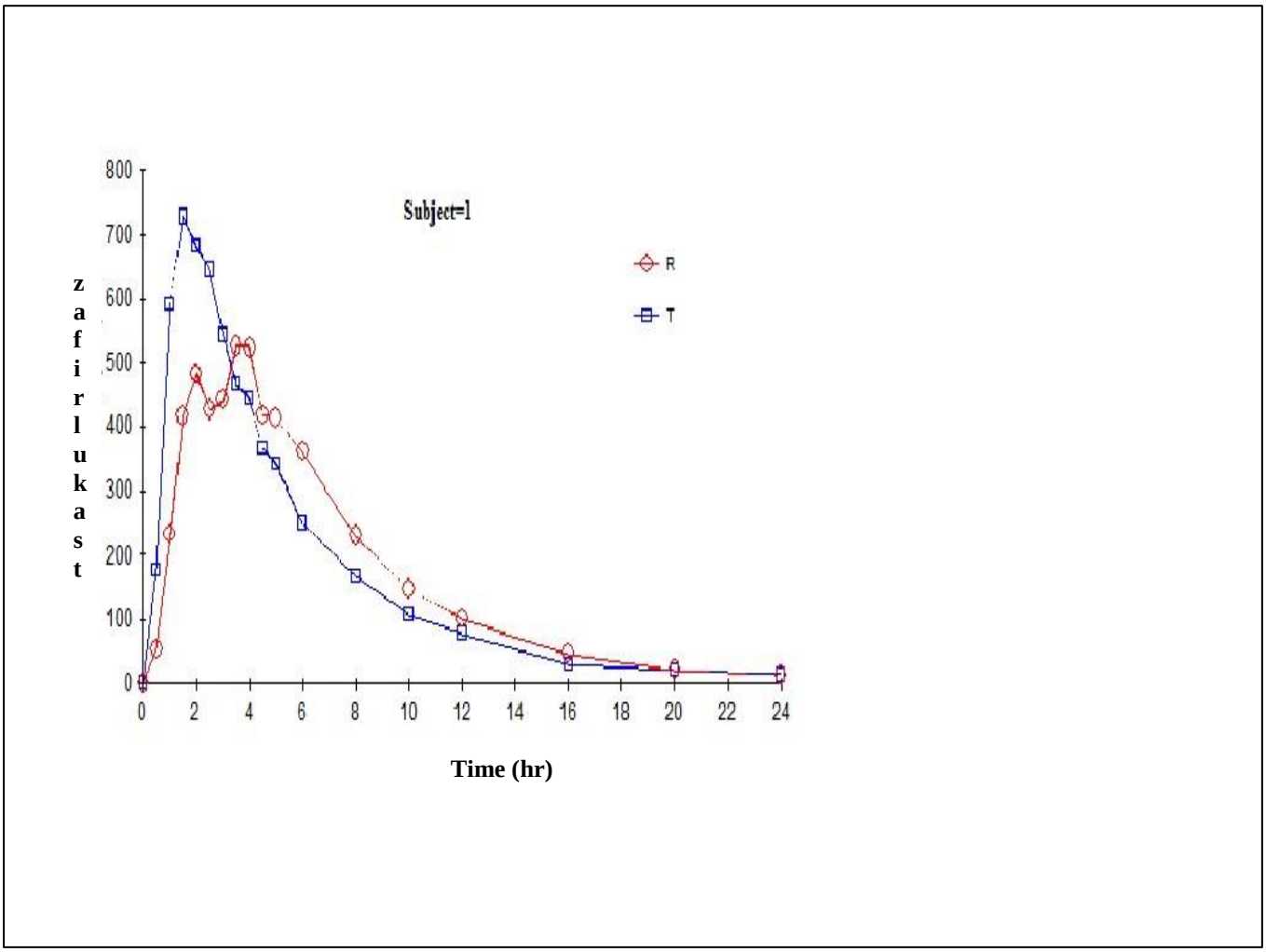
Statistical analysis software (SAS®) - includes a vast range of statistics for general statistical analysis which includes multiple linear regression, correlation, ANOVA (analysis of variance), chi-square, Fisher, McNemar, Wilcoxon, Mann-Whitney, Friedman, Kruskal Wallis, Shapiro-Wilk normality tests, histograms, summary statistics and many more.

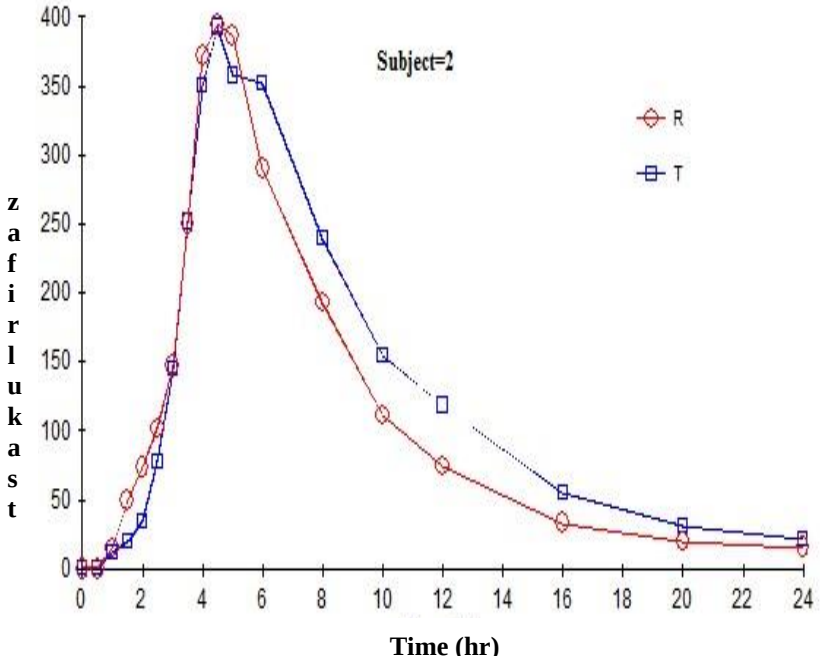
With the help of these softwares the PK parameters and statistical results are calculated for the data obtained after the completion of the study[18].

Results and Discussion

The Zafirlukast plasma concentration of 12 individual subjects in the given study reports as follows:

Linear plot mean of individual plasma Zafirlukast conc vs time under fasting conditions

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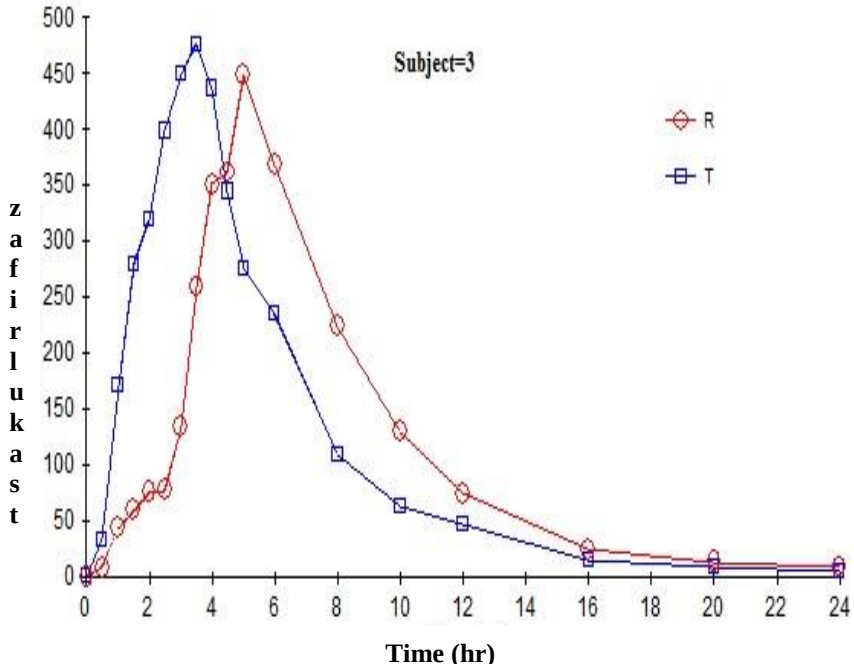
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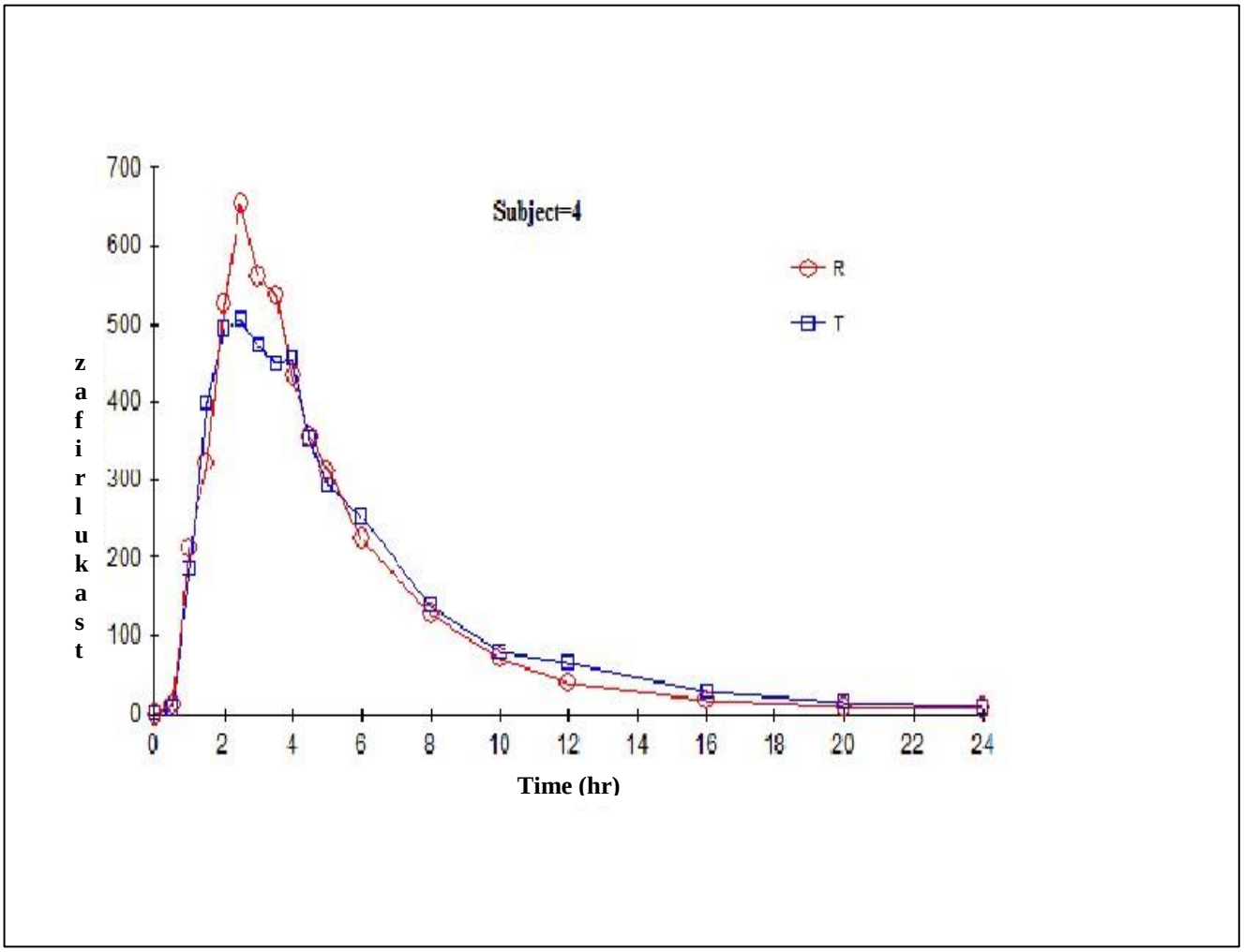
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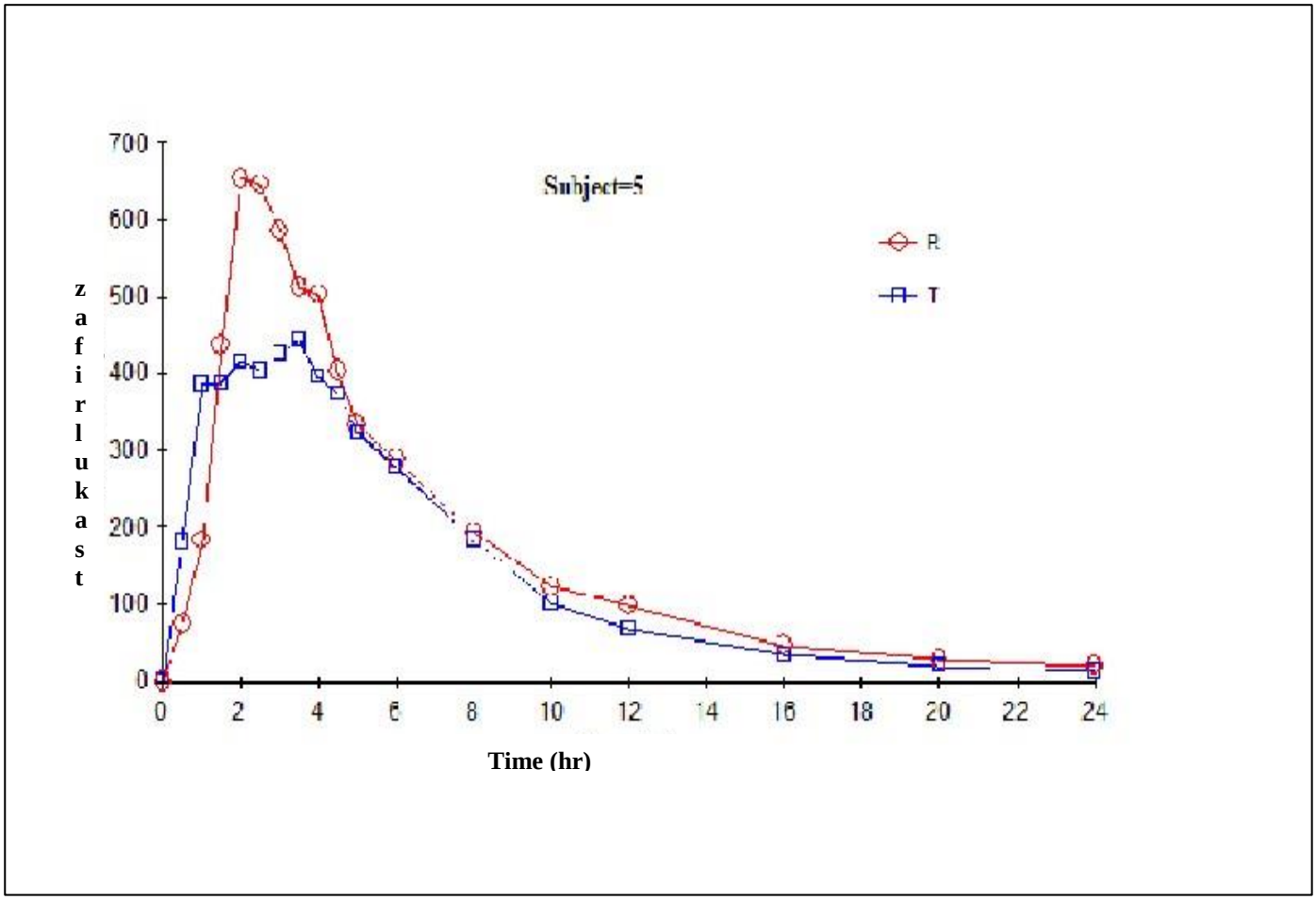
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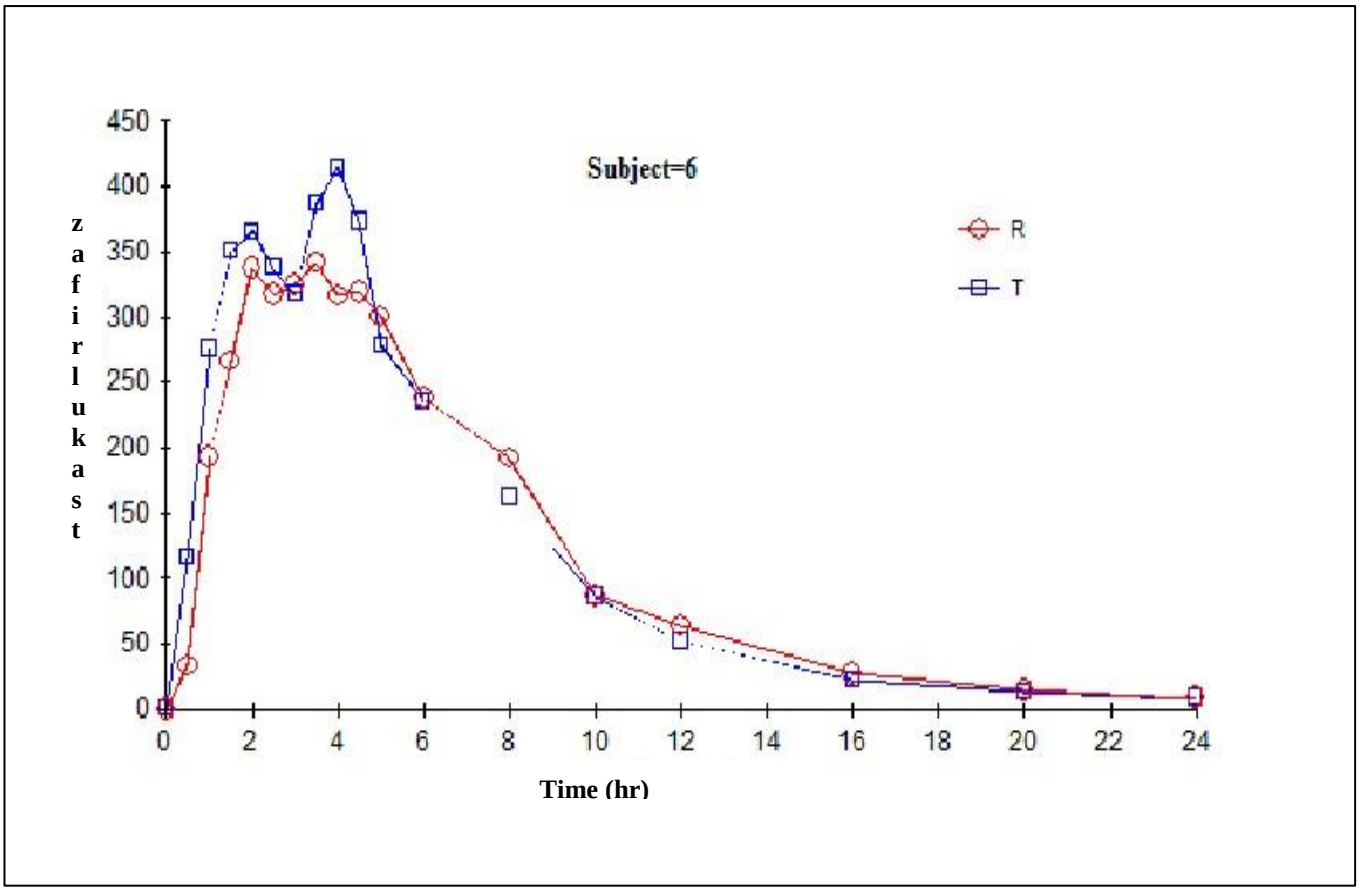
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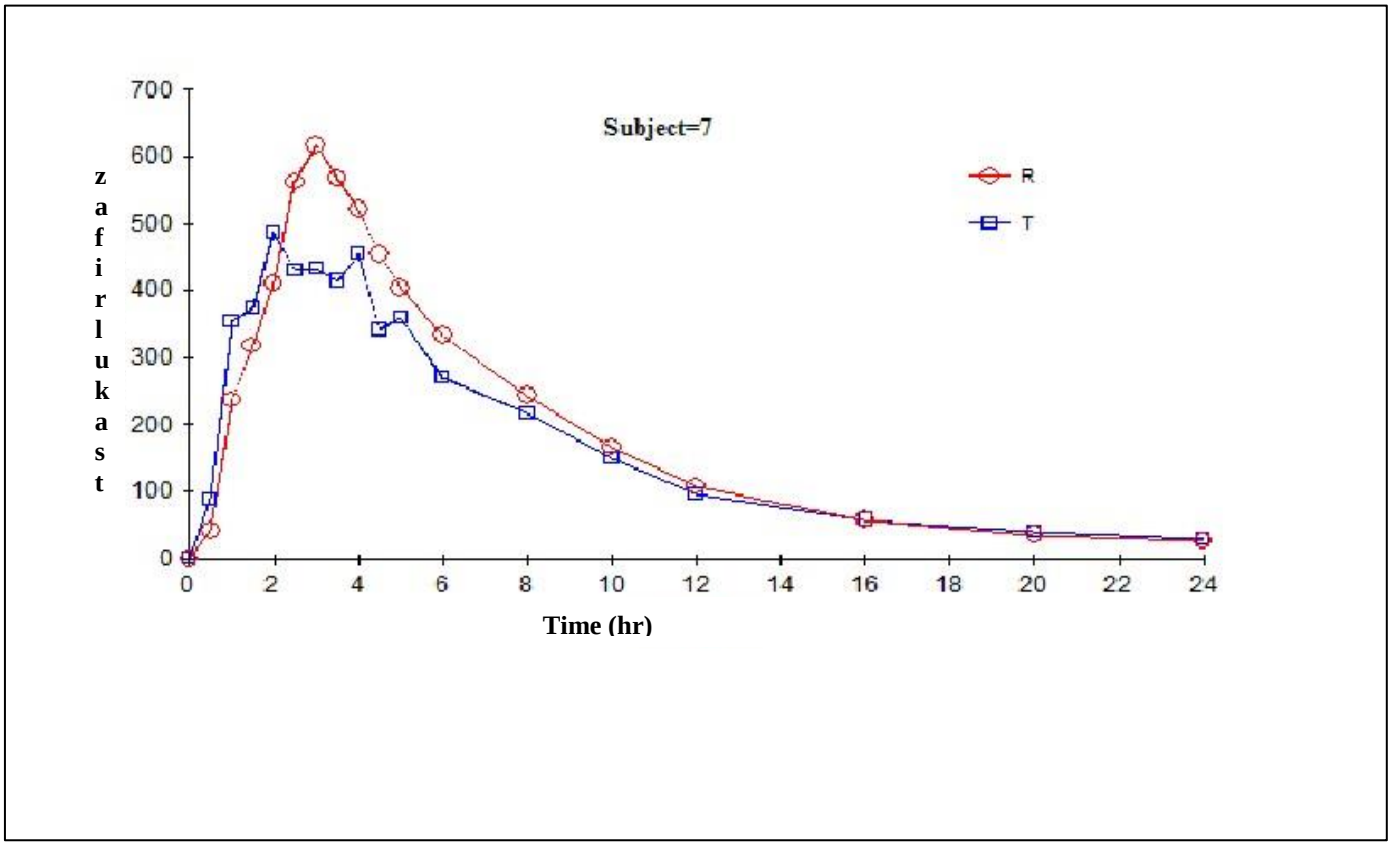
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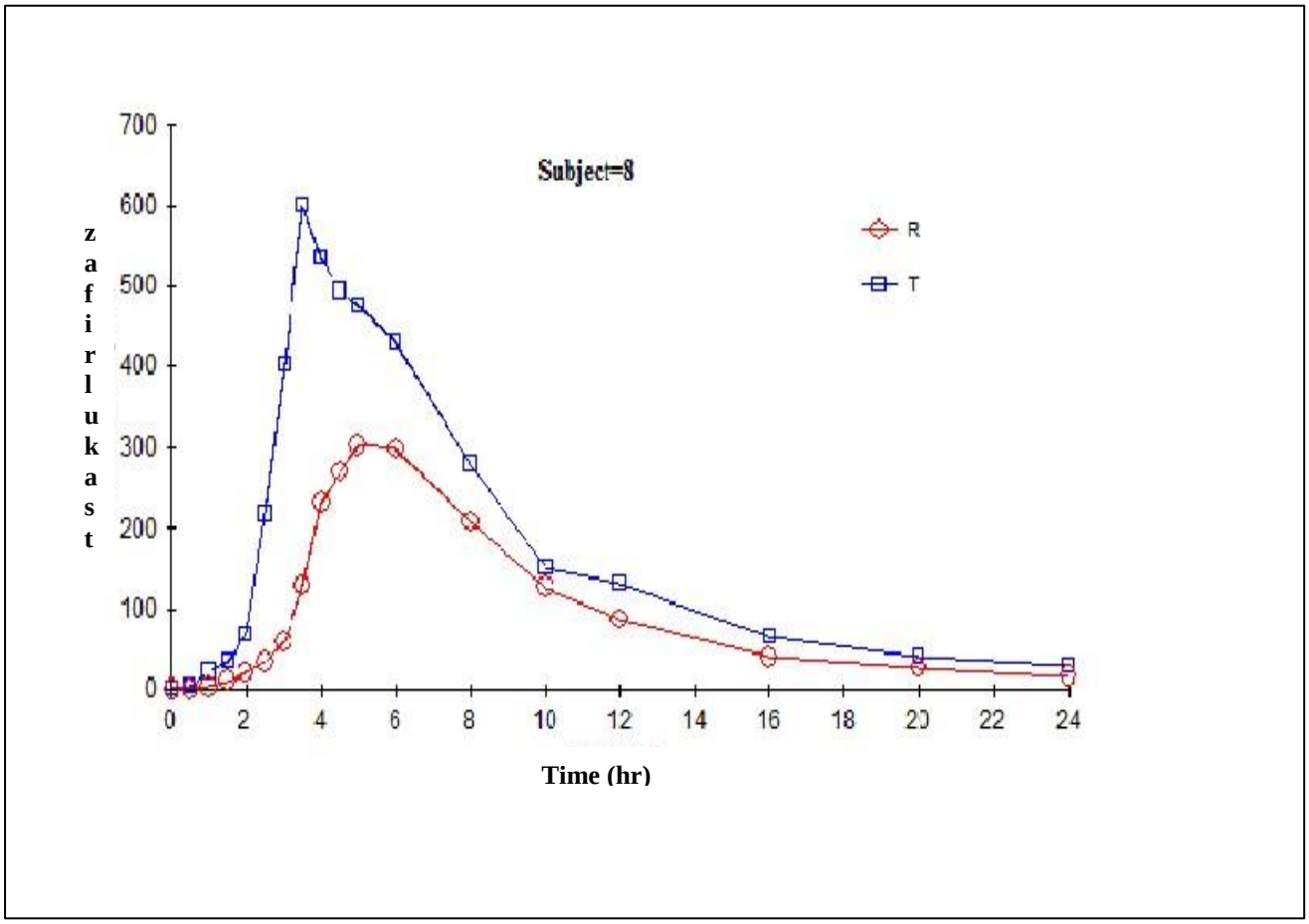
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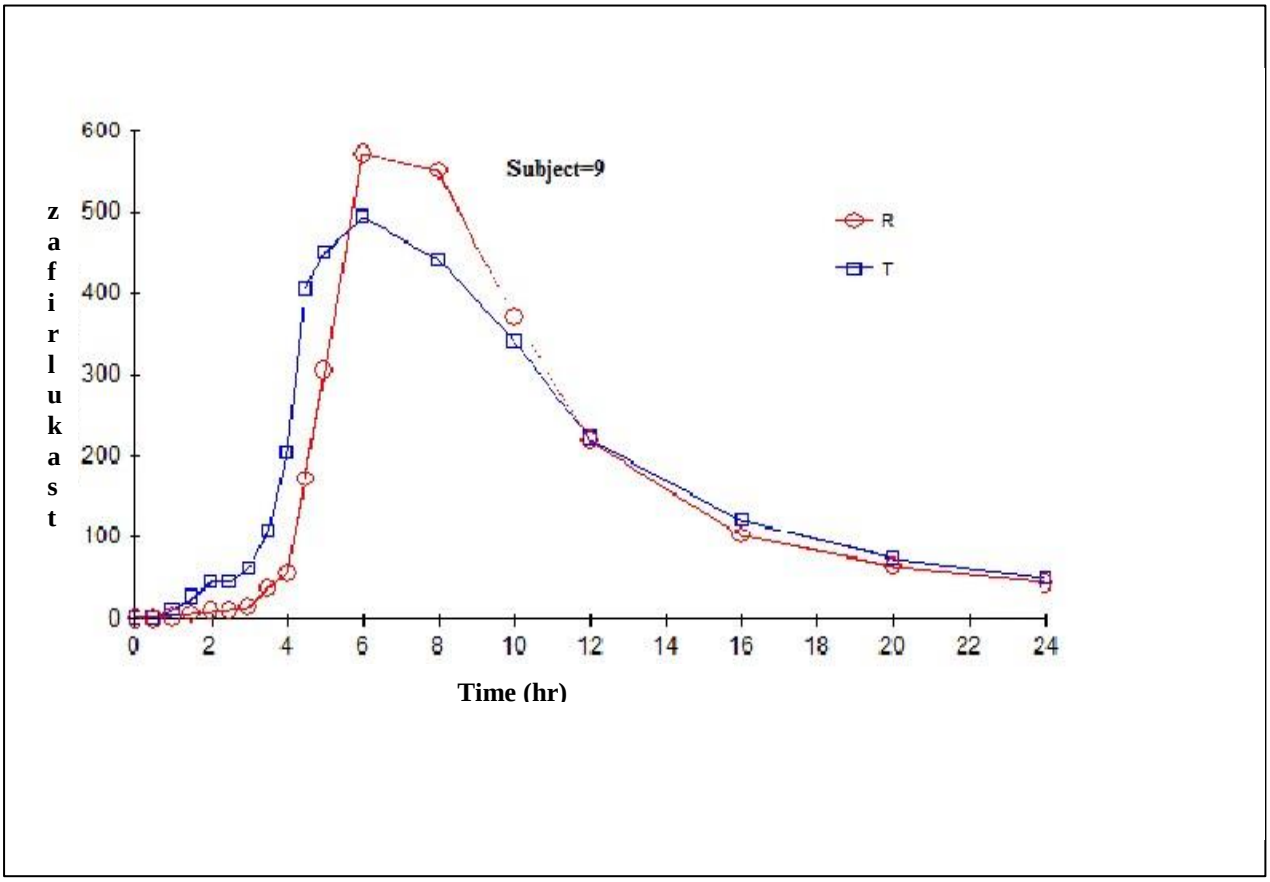
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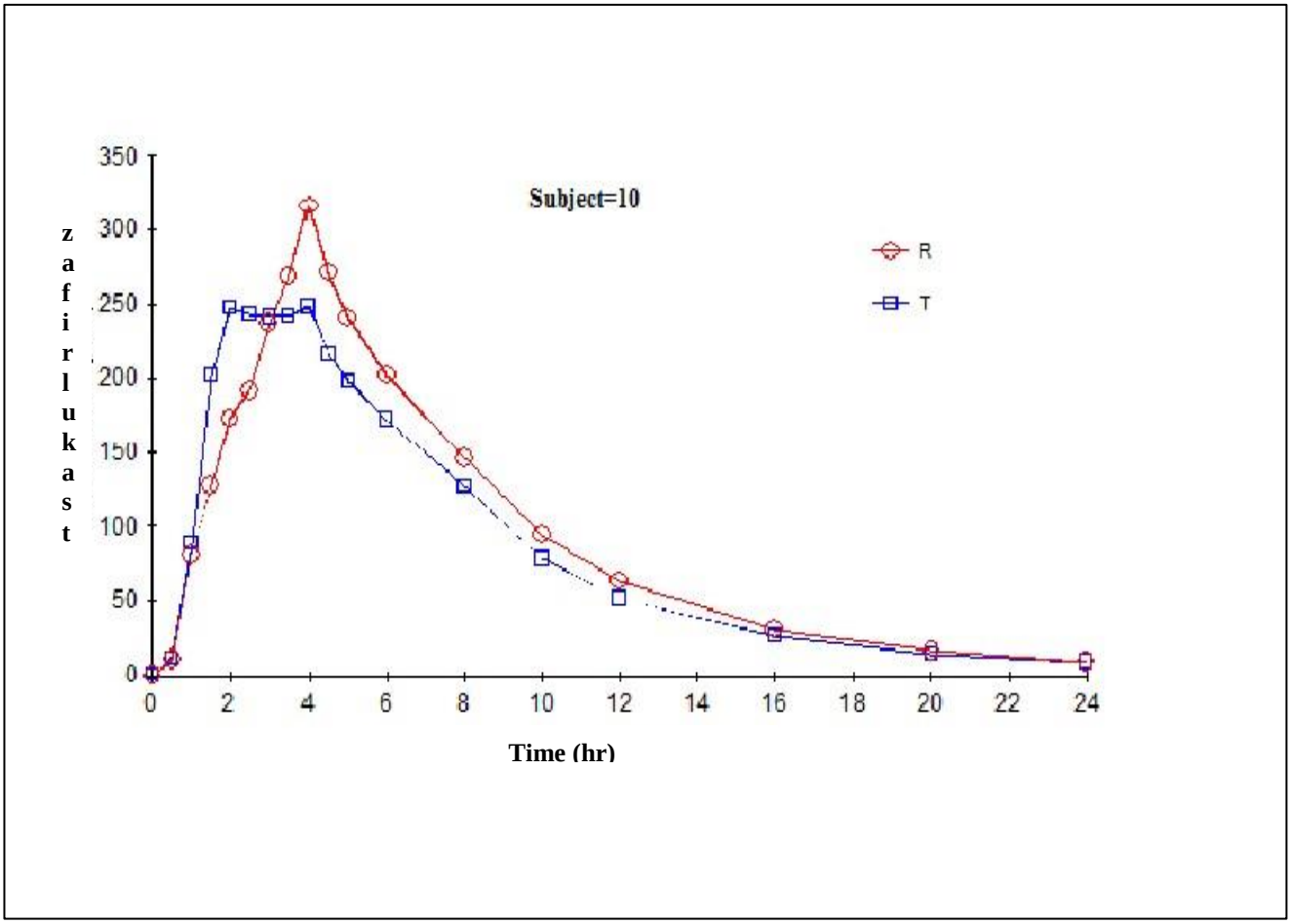
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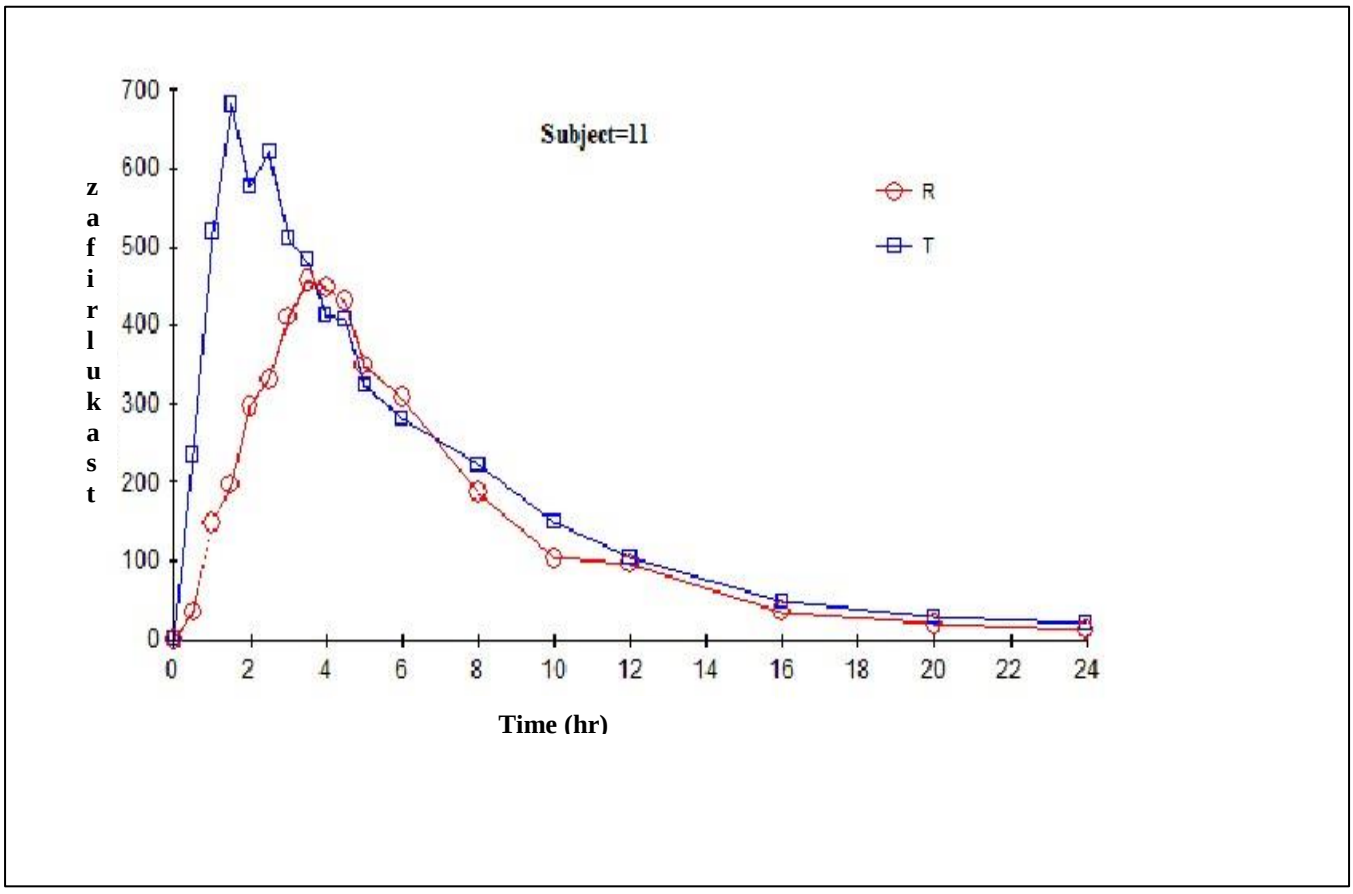
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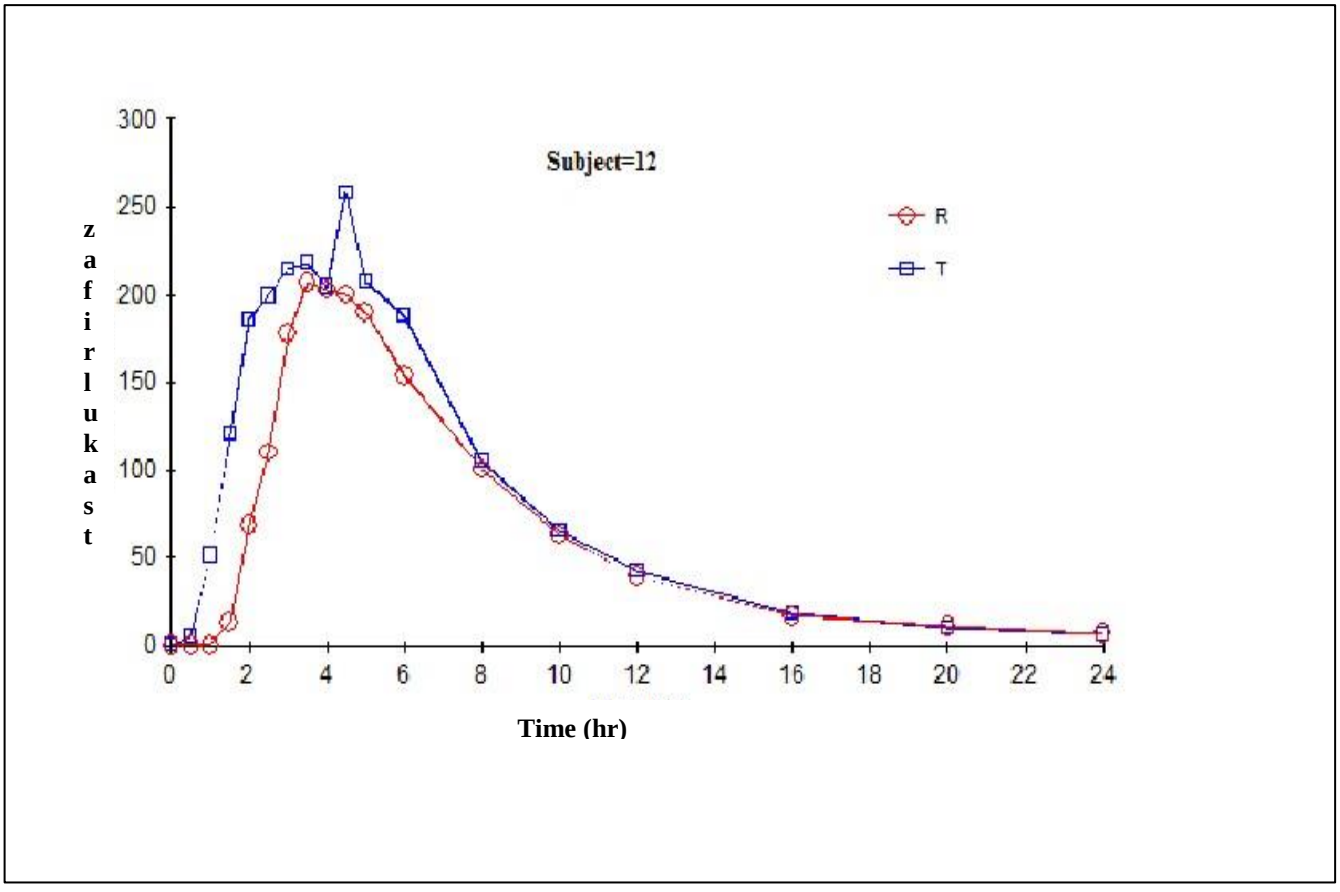
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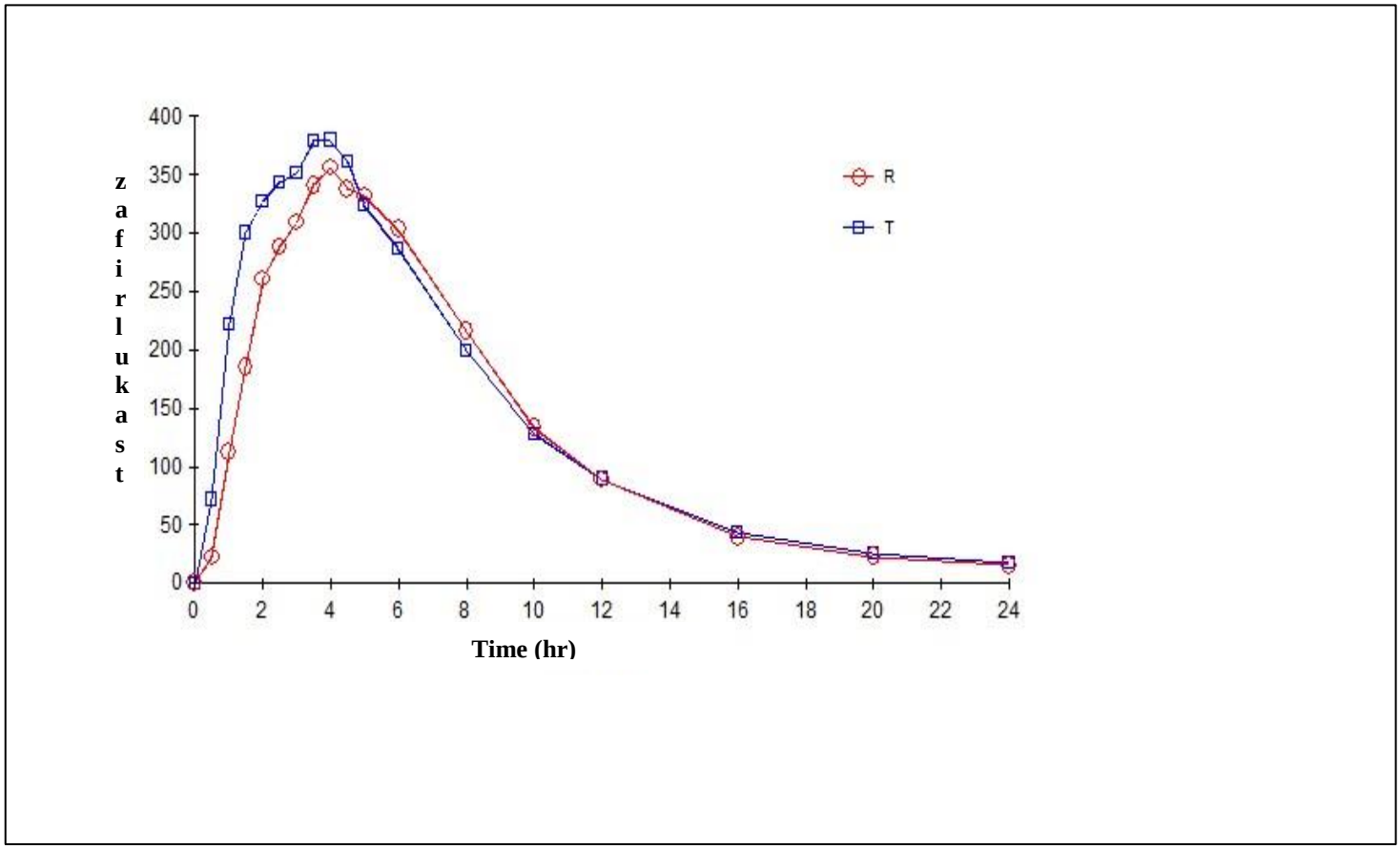
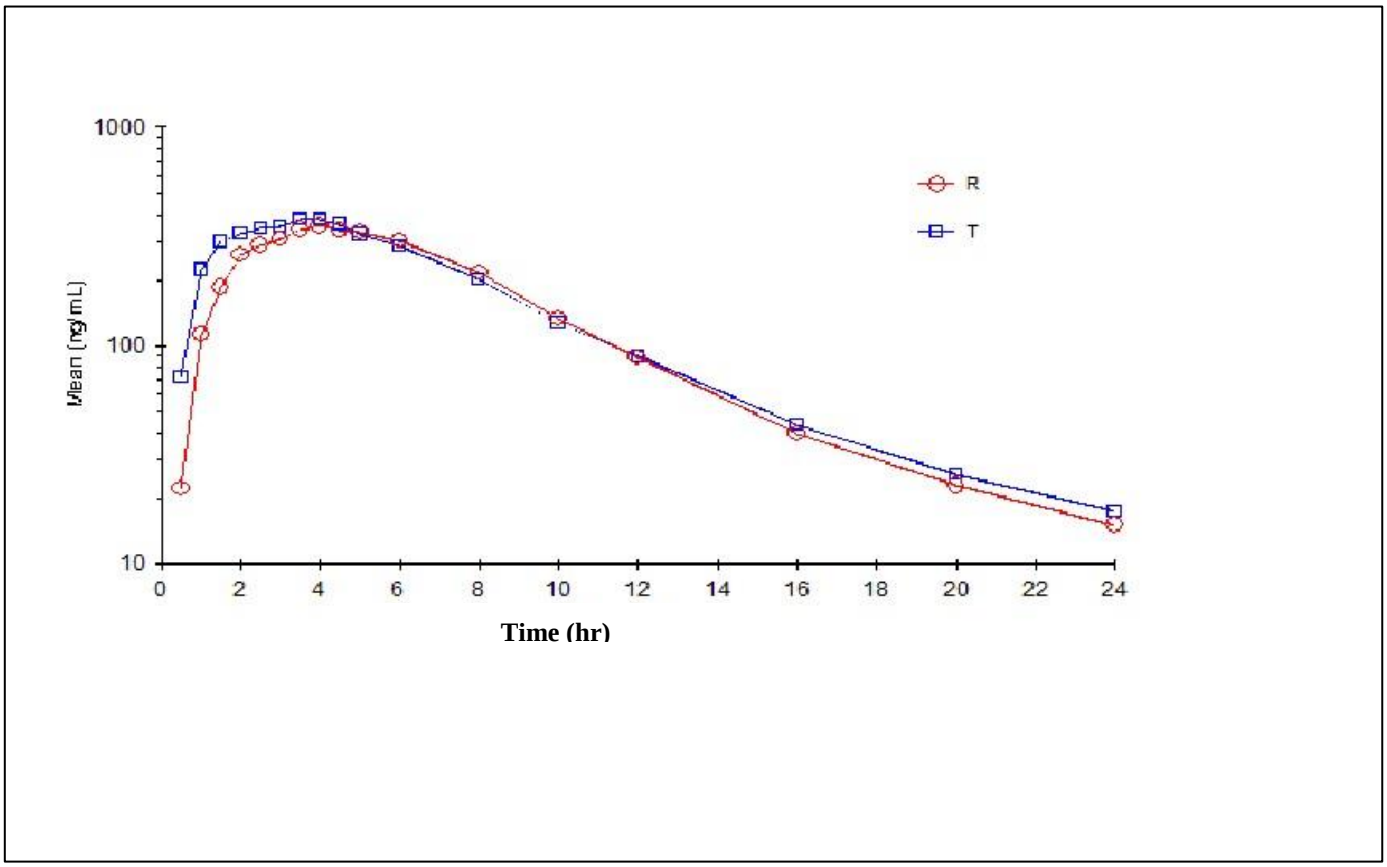
Time (hr)

Linear mean plot of plasma Zafirlukast conc vs time under fasting conditions



Semilog mean plot of plasma Zafirlukast conc vs time under fasting conditions



				Zafirlukast																
				Time																
Treatment	Subject	Period	Sequence	0.00	0.50	1.00	1.50	2.00	2.50	3.00	3.50	4.00	4.50	5.00	6.00	8.00	10.00	12.00	16.00	20.00	24.00
R	1	2	TR	0.00	53.48	231.93	416.88	482.62	425.54	441.07	526.11	522.68	418.10	413.07	361.43	228.96	146.70	100.66	47.08	21.07	11.95
	2	1	RT	0.00	0.00	14.98	49.40	73.19	101.46	147.27	250.22	371.75	394.68	386.31	290.12	193.11	110.81	74.06	32.99	19.80	15.33
	3	2	TR	0.00	8.02	43.16	59.18	75.27	77.13	133.70	258.89	350.84	361.75	448.30	368.20	224.07	129.44	74.09	24.12	13.40	8.15
	4	1	RT	0.00	11.67	211.56	321.43	525.91	653.63	561.08	537.02	435.30	356.56	311.70	223.75	127.55	71.93	39.89	17.23	8.52	6.73
	5	2	TR	0.00	74.09	182.59	436.97	653.66	646.32	585.61	512.70	503.42	403.35	332.84	288.75	193.27	123.30	98.50	46.51	27.79	20.41
	6	1	RT	0.00	33.06	192.57	265.93	336.77	317.09	324.91	341.91	316.12	319.13	299.96	237.78	191.80	86.67	63.24	26.86	14.27	8.42
	7	1	RT	0.00	41.03	236.59	318.31	411.17	562.05	617.86	567.97	521.19	453.46	403.18	332.92	242.88	166.44	106.73	57.76	34.86	25.35
	8	2	TR	0.00	0.00	2.68	9.85	20.11	33.22	58.99	129.92	231.41	270.65	302.76	298.56	208.43	127.73	87.64	39.02	25.60	15.26
	9	1	RT	0.00	0.00	2.10	4.94	8.27	9.69	13.46	36.89	54.56	171.36	304.34	569.75	549.35	370.25	217.88	101.11	63.08	43.07
	10	2	TR	0.00	10.38	80.53	127.28	173.15	192.30	236.62	268.75	316.04	271.51	240.65	202.69	145.81	94.31	63.42	31.29	16.62	8.98
	11	1	RT	0.00	34.34	148.54	196.81	297.21	331.22	410.37	456.80	447.86	432.06	348.84	308.03	186.54	103.40	96.32	34.85	19.13	11.45
	12	2	TR	0.00	0.00	0.00	12.82	68.11	109.56	177.41	206.98	202.24	200.20	189.54	153.99	99.93	62.93	39.35	16.65	10.69	6.81
			N	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
			Mean	0.00	22.17	112.27	184.98	260.45	288.27	309.03	341.18	356.12	337.73	331.79	303.00	215.98	132.83	88.48	39.62	22.90	15.16
			SD	0.000	24.705	97.394	162.544	221.496	237.687	211.642	176.403	143.026	91.976	73.914	105.714	113.158	80.554	46.557	22.926	14.697	10.480
			Min	0.00	0.00	0.00	4.94	8.27	9.69	13.46	36.89	54.56	171.36	189.54	153.99	99.93	62.93	39.35	16.65	8.52	6.73
			Median	0.00	11.03	114.54	162.05	235.18	254.70	280.77	305.33	361.30	359.16	322.27	294.34	193.19	117.06	80.87	33.92	19.47	11.70
			Max	0.00	74.09	236.59	436.97	653.66	653.63	617.86	567.97	522.68	453.46	448.30	569.75	549.35	370.25	217.88	101.11	63.08	43.07
			CV%	Missing	111.42	86.75	87.87	85.04	82.45	68.49	51.70	40.16	27.23	22.28	34.89	52.39	60.65	52.62	57.86	64.17	69.13
T	1	1	TR	0.00	177.62	592.10	727.76	680.82	644.39	541.38	466.49	443.10	365.55	340.78	249.58	167.54	106.92	77.01	30.18	19.04	12.28
	2	2	RT	0.00	0.00	11.33	19.90	34.32	77.47	144.52	251.70	349.81	392.61	357.47	351.97	239.65	154.63	118.33	54.56	30.42	21.28
	3	1	TR	0.00	33.13	170.49	279.19	319.00	398.56	449.12	476.59	436.70	344.48	275.87	235.04	109.07	62.26	46.63	14.83	7.76	5.05
	4	2	RT	0.00	7.37	184.94	399.74	493.31	505.44	472.97	450.98	455.79	353.19	293.37	251.96	137.72	79.64	64.80	27.63	14.16	8.86
	5	1	TR	0.00	181.77	386.67	388.92	416.01	403.88	426.03	444.10	395.83	374.91	322.61	278.44	184.25	101.24	68.37	33.41	19.32	13.08
	6	2	RT	0.00	115.27	275.67	350.03	365.68	337.36	318.61	386.60	414.31	373.08	278.68	235.75	161.82	85.89	51.89	22.08	12.44	8.35
	7	2	RT	0.00	88.43	354.08	374.63	485.82	430.18	433.82	415.06	454.79	342.10	360.22	271.03	217.37	149.05	95.67	58.05	40.57	28.48
	8	1	TR	0.00	3.68	21.56	33.67	68.95	218.33	402.26	600.97	533.66	493.58	475.27	430.17	280.16	152.77	131.53	64.93	39.31	29.46
	9	2	RT	0.00	0.00	9.47	25.44	43.83	44.25	60.37	105.92	203.40	403.94	451.41	493.86	441.58	339.40	220.39	120.40	73.10	48.27
	10	1	TR	0.00	11.93	88.68	202.28	247.07	242.97	241.15	242.01	248.03	216.86	198.39	172.37	126.87	78.36	51.53	26.48	13.86	9.33
	11	2	RT	0.00	235.32	518.99	681.70	577.15	621.40	510.99	482.73	414.01	408.71	323.21	281.83	222.69	148.85	103.83	49.10	28.14	19.41
	12	1	TR	0.00	5.34	51.37	120.36	185.46	199.57	214.27	218.36	204.36	258.14	207.11	188.23	104.77	65.62	42.46	18.48	10.26	6.53
			N	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
			Mean	0.00	71.66	222.11	300.30	326.45	343.65	351.29	378.46	379.48	360.60	323.70	286.69	199.46	127.05	89.37	43.34	25.70	17.53
			SD	0.000	85.703	202.992	237.721	215.108	194.242	153.851	142.488	106.639	70.777	83.438	94.731	93.660	75.576	50.536	29.334	18.527	12.705
			Min	0.00	0.00	9.47	19.90	34.32	44.25	60.37	105.92	203.40	216.86	198.39	172.37	104.77	62.26	42.46	14.83	7.76	5.05
			Median	0.00	22.53	177.72	314.61	342.34	367.96	414.15	429.58	414.16	369.32	322.91	261.50	175.90	104.08	72.69	31.80	19.18	12.68
			Max	0.00	235.32	592.10	727.76	680.82	644.39	541.38	600.97	533.66	493.58	475.27	493.86	441.58	339.40	220.39	120.40	73.10	48.27

		Tmax		Cmax		AUClast		AUCINF_obs		Lambda_z		HL_Lambda_z		AUC_%Extrap_o		Rsq	
		Treatment		Treatment		Treatment		Treatment		Treatment		Treatment		Treatment		Treatment	
Subject	Sequence	R	T	R	T	R	T	R	T	R	T	R	T	R	T	R	T
1	TR	3.50	1.50	526.11	727.76	3960.95	3950.95	4023.44	4060.20	0.1912	0.1124	3.62	6.17	1.55	2.69	0.9976	0.9998
2	RT	4.50	4.50	394.68	392.61	2498.23	2962.92	2583.69	3096.98	0.1794	0.1587	3.86	4.37	3.31	4.33	0.9716	0.9910
3	TR	5.00	3.50	448.30	476.59	2668.17	2596.10	2728.26	2617.73	0.1356	0.2336	5.11	2.97	2.20	0.83	0.9977	0.9779
4	RT	2.50	2.50	653.63	505.44	3011.57	3073.83	3040.65	3125.35	0.2314	0.1720	3.00	4.03	0.96	1.65	0.9729	0.9892
5	TR	2.00	3.50	653.66	444.10	3948.77	3381.85	4069.01	3453.55	0.1697	0.1824	4.08	3.80	2.96	2.08	0.9823	0.9838
6	RT	3.50	4.00	341.91	414.31	2733.41	2836.81	2791.47	2905.51	0.1450	0.1216	4.78	5.70	2.08	2.36	0.9973	0.9893
7	RT	3.00	2.00	617.86	485.82	4226.59	3837.46	4384.69	4157.41	0.1603	0.0890	4.32	7.79	3.61	7.70	0.9860	1.0000
8	TR	5.00	3.50	302.76	600.97	2277.57	3926.37	2407.60	4114.23	0.1174	0.1568	5.91	4.42	5.40	4.57	0.9965	0.9808
9	RT	6.00	6.00	569.75	493.86	4469.26	4621.33	4873.01	5043.82	0.1067	0.1143	6.50	6.07	8.29	8.38	0.9963	0.9972
10	TR	4.00	4.00	316.04	248.03	2202.90	2022.91	2260.45	2077.57	0.1560	0.1707	4.44	4.06	2.55	2.63	0.9999	0.9923
11	RT	3.50	1.50	456.80	681.70	3208.94	4293.00	3291.24	4410.43	0.1391	0.1653	4.98	4.19	2.50	2.66	0.9980	0.9926
12	TR	3.50	4.50	206.98	258.14	1428.55	1762.47	1489.49	1812.69	0.1118	0.1300	6.20	5.33	4.09	2.77	1.0000	0.9943
	N	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	Mean	3.83	3.42	457.37	477.44	3052.91	3272.17	3161.92	3406.29	0.1536	0.1506	4.73	4.91	3.29	3.55	0.9913	0.9907
	SD	1.135	1.346	148.959	145.783	932.091	889.537	993.516	978.740	0.03608	0.03921	1.067	1.333	1.967	2.330	0.01044	0.00703
	Min	2.00	1.50	206.98	248.03	1428.55	1762.47	1489.49	1812.69	0.1067	0.0890	3.00	2.97	0.96	0.83	0.9716	0.9779
	Median	3.50	3.50	452.55	481.21	2872.49	3227.84	2916.06	3289.45	0.1505	0.1578	4.61	4.39	2.75	2.68	0.9969	0.9917
	Max	6.00	6.00	653.66	727.76	4469.26	4621.33	4873.01	5043.82	0.2314	0.2336	6.50	7.79	8.29	8.38	1.0000	1.0000
	CV%	29.60	39.38	32.57	30.53	30.53	27.18	31.42	28.73	23.48	26.04	22.55	27.16	59.77	65.57	1.05	0.71

Safety and Efficacy

The safety criteria included in the study were presence or absence of adverse events, adverse drug reactions and also any changes in the physical or biochemical parameters after completion of the study when compared with pre study values.

During post study assessment vitals and medical examination for all the subjects participating in the study were normal, however a few of them had abnormal values in the medical reports. These cases were reported as adverse events. The following table provides a list of adverse events occurred in the subjects and the treatment given to them.

Subject Number*	Period	Last Treatment Received	Adverse Event
S7	Post study	T	Increased SGOT (100 U/L)
			Increased SGPT (116 U/L)

- From the adverse events profile and tolerability of the subjects, it appeared that the test product was safe and tolerable as that of reference product.
- No deaths and serious adverse events were reported during the entire duration of the study

ABNORMAL LABORATORY VALUE LISTING (Each Subject)

Listing of Abnormal Hematology Laboratory Values

Parameter	Subject Number	Pre/Post study		Remarks
		S	P	
Total RBC (mill/cumm)	S1	6.25 H	--	CNS
	S5	--	4.4 L	CNS
	S9	---	6.0 H	CNS
	S7	4.1 L	4.3 L	CNS
	S11	6.06 H	4.8 L	CNS
Haemoglobin (gm/dL)	S1	--	12.5 L	CNS
	S5	12.3 L	12.2 L	CNS
	S12	--	12.4 L	CNS
Total WBC (cells/cumm)	S3	11700 H	--	CNS

S: Pre-study (screening) evaluation; P: Post study evaluation.

WBC: Leukocytes;

RBC: Erythrocytes

^{L}: Lower than normal range; ^{H}: Higher than normal range.

CNS: Clinically not significant.

Listing of Abnormal Biochemistry Laboratory Values

Parameter	Subject Number	Pre/Post Study		Remarks
		S	P	
Uric Acid(mg/dL)	S2	7.5 ^{H}	--	CNS
Urea (mg/dL)	S2	44 ^{H}	--	CNS
	S4	14 ^{L}	--	CNS
	S9	--	16 ^{L}	CNS
Total Protein (g/Dl)	S1	8.7 ^{H}	--	CNS
	S3	8.6 ^{H}	--	CNS
	S7	8.5 ^{H}	--	CNS
Total cholesterol(mg/dL)	S2	211 ^{H}	--	CNS

	S7	220H	--	CNS
Calcium(mg/dL)	S1	10.7 ^{H}	--	CNS
Sodium(mmol/L)	S5	134 ^{L}	--	CNS
SGOT	S7	--	100 ^{H}	CS
SGPT	S2	--	46 ^{H}	CNS
	S7	--	116 ^{H}	CS

S: Pre-study (screening) evaluation; P: Post study evaluation.

S: Pre-study (screening) evaluation; P: Post study evaluation.

SGOT: Serum Glutamate Oxaloacetate Transaminase

SGPT: Serum Glutamate Pyruvate Transaminase

^{L}: Lower than normal range; ^{H}: Higher than normal range.

CNS: Clinically not significant.

Calibration Curve Standards Data

A total of 25 CCs were run for the bioanalysis of subject plasma samples containing drug Zafirlukast . The slopes, intercepts and goodness of fit were determined by Linear regression analyses using the ratios of drug / internal standard peak areas of the CC standards. A weighting factor of $1/x^2$ ($1/$ concentration^2) was used in the calculation of the linear regression line and the concentrations of all the study samples and QC samples were calculated by Analyst 1.4.1 software system. The Regression coefficient (r^2) was greater than 0.99 for drug Zafirlukast throughout the study.

Quality Control Samples Data

A total of 116 sets of QC samples at 3 different concentrations (LQC, MQC & HQC) of drug Zafirlukast were analyzed, interspersed with the subject samples.

Subject Sample Analyses Data

A total of 12 subject samples were analysed as per the validated method. The QC samples were dispersed throughout the subject run to control and monitor precision of batch inter-assay variation.

Stability of the stock solutions in the matrix

The stock solutions of the drug as well as the reference standards were found to be stable. Benchtop stability, Freeze-thaw stability and long term stabilities were determined.Long-term stability was determined for a period of 3 months.

Pharmacokinetic parameters and statistical Data

Pharmacokinetic parameters and statistical analyses were calculated from subject concentrations using validated WinNonlin® Version 5.1 and SAS® Version 9.1 software procedures, respectively.

Pharmacokinetic Data

The following table summarizes the pharmacokinetic results obtained for test and reference formulations of drug Zafirlukast in the fasting condition.

PK PARAMETERS	DRUG	
	ZAFIRLUKAST (TEST)	ACCOLATE (REFERENCE)
C_{max} (µg/mL) **Mean** **SD** **CV%**	477.44 148.959 32.57	457.37 145.783 30.53
AUC_{0-t} (µg.hr/mL) **Mean** **SD** **CV%**	3272.17 889.537 27.18	3052.91 932.091 30.53

$AUC_{0-\infty}$ (µg.hr/mL)		
Mean	3406.29	3161.92
SD	978.740	993.51
CV%	28.73	31.42
AUC_Extrapolated (%)		
Mean	3.55	3.29
SD	2.330	1.960
CV%	65.57	59.77
T_{max} (hr)		
Mean	3.42	3.83
SD	1.346	1.135
Median	3.50	3.50
CV%	39.38	29.60
K_{el} (hr^{-1})		
Mean	0.1506	0.1536
SD	0.03921	0.03608
CV%	26.04	23.48

ZAFIRLUKAST 90% CONFIDENCE INTERVAL DATA

	Test T			
Dependent	Ratio[%Ref]	CI 90 Lower	CI 90 Upper	Power
Ln(AUCINF_obs)	108.46	97.59	120.53	0.96
Ln(AUClast)	108.15	97.09	120.48	0.96
Ln(Cmax)	105.21	88.42	125.19	0.69

No statistically significant differences were observed for sequence and formulation effects for ln-transformed $C_{max,}$ AUC_{0-t} & $AUC_{0-\infty}$ data of the Test and Reference Product.

Intra-subject Variability:

The coefficients of variation (CV%) corresponding to intra-subject variability for C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ were 30.53 %, 27.18 % and 28.73 %, respectively, which were found to be less than 30%.

Power:

The power values obtained for C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ were 88.42 %, 97.09 % and 97.59 %, respectively, which were greater than 80.00 % the desired power to support the bioequivalence test, and hence considered to be adequate for supporting bioequivalence conclusions.

CONCLUSION

All the study procedures followed were in compliance with the protocol and the ICH-GCP guidelines, Declaration of Helsinki and Schedule Y.

From the results obtained, in both the fast and the fed study, it is observed that there is no significant difference in the pharmacokinetic parameters, indicating that the bioavailability of drug XY is not affected by food.

From the analyses of pharmacokinetic and statistical results it was inferred that, for the ln-transformed data, the 90 % confidence interval about the test to reference ratio of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ of drug XY were falling within the bioequivalence acceptance range of 80.00 % - 125.00 %, which demonstrates the bioequivalence of test formulation 'ZAFIRLUKAST' with reference formulation 'SINGULIAR' under fasting conditions.

From the clinical data it can be concluded that the study objectives like the safety and efficacy of the test product has been achieved.

Based on clinical, pharmacokinetic and statistical data obtained from healthy, adult, male, human subjects under fasting conditions, it was concluded that a single dose of test formulation 'ZAFIRLUKAST' containing drug 1000 mg was found to be safe and bioequivalent to the reference formulation 'ACCOLATE' containing drug 1000 mg as 90 % confidence interval for the ratios of means of test and reference parameters such as ln-transformed C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ of drug is within the bioequivalence acceptance range of 80.00% – 125.00 %.

GLOSSARY

1. **Adverse Event:** An adverse event is any untoward medical occurrence in clinical investigation subject administered a pharmaceutical product and that does not necessarily have a causal relationship with this treatment.

2. **Analysis of Variance (ANOVA):** ANOVA is a statistical technique to identify sources of variance and estimate the degree of variability. In most bioavailability studies, there are three readily identified sources of variance namely formulation (Treatment), subject and period; hence it is a 3-way ANOVA.

3. **Area under the curve (AUC):** Area under the curve is the total area under the biological fluid (serum, blood, etc.) concentration-time curve as determined by the Trapezoidal rule.

4. **AUC_Extrapolated (%):** Calculated as {1 - (AUC0-t / AUC0-∞)} x 100. The mean AUC_Extrapolated (%) should be ≤ 20%.

5. **Bioavailability studies:** It involves the determination of 'Drug' concentration in the blood or urine. Concern with how quickly and how much of a 'Drug' appears in the blood after a specific dose is administered.

6. **C_{max}:** This is the maximum 'Drug' concentration achieved in systemic circulation following 'Drug' administration

7. **Good Clinical Practice:** A standard for the design, conduct, performance, monitoring, auditing, recording, analyses and reporting of clinical trials that provides assurance that

the data and reported results are credible and accurate, and that the rights, integrity, and confidentiality of trial subjects are protected.

8. **Informed Consent:** A process by which a subject voluntarily confirms his or her willingness to participate in a particular trial, after having been informed of all aspects of the trial are relevant to the subject's decision to participate. Informed consent is documented by means of a written, signed, and dated informed consent form.

9. **Investigational Product:** A pharmaceutical form of an active ingredient being tested or used as a reference in a clinical trial, including a product with a marketing authorization when used or assembled (formulated or packaged) in a way different from the approved form, or when used for an unapproved indication, or when used to gain further information about an approved use.

10. **K_{el}:** Apparent first-order terminal elimination rate constant calculated from a semi-log plot of the plasma concentration versus time curve, using the method of least square regression.

11. **Protocol:** A document that describes the objective(s), design, methodology, statistical consideration, and organization of a trial. The protocol usually also give the background and rationale for the trial, but these could be provided in other protocol referenced documents. Throughout the ICH-GCP Guidance, the term protocol refers to protocol and protocol amendments.

12. **Quality Assurance:** All those planned and systematic action that are established to ensure that the trial is performed and the data are generated, documented (recorded), and reported in compliance with GCP and the applicable regulatory requirements(s).

13. **Quality Control:** The operational techniques and activities undertaken within the quality assurance system to verify that the requirements for quality of the trial related activities have been fulfilled.

14. **Randomization:** The process of assigning trial subject to treatment groups using an element of chance to determine the assignments in order to reduce bias.

15. **Sponsor-Investigator:** An individual who both initiate and conducts, alone or with others, a clinical trial and under whose immediate direction the investigational product is administered to, dispensed to, or used by a subject. The term does not include any person other than an individual (e.g. it does not include a corporation or an agency). The obligations of a sponsor-investigator include both those a sponsor and those of an investigator.

16. **T_{max}:** It is the time required to achieve maximum 'Drug' concentration in systemic circulation

17. **$t_{1/2}$:** Terminal half-life as determined by quotient 0.693/ Kel

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